

REVIEW

CD4⁺ T cell epitope identification: applications to allergy

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Allergy as an immunological disease

Type I hypersensitivity, or IgE-mediated allergic reaction, is the result of cross-linking Fc receptor-bound IgE on the surface of mast cells. Cross-linked IgE signals the release of mediators that result in the clinical manifestations of what is termed an allergic reaction: rhinitis, urticaria, conjunctivitis, bronchospasm, diarrhea, vomiting and hypotension. Mast cells themselves do not generate IgE antibody. IgE is produced by antigen-specific B cells activated by CD4⁺ T helper cells in the presence of the cytokines IL-4 and/or IL-13 [1, 2]. Signalling via IL-4 and/or IL-13 promotes immunoglobulin isotype switching, a process where the antibody specificity remains the same, but the isotype of the antibody is changed via DNA-level recombination. Switching to IgE is often achieved in a step-wise process via first switching to IgG4 in humans, and IgG1 in mice. Often, IgG4 and IgG1 isotypes will be followed as surrogate markers for allergic antibodies.

Activation of antigen-specific B cells to switch isotypes is mediated by CD4⁺ helper T cells [3]. CD4 T cells with a specificity for the same antigen as specific B cells will form a cognate interaction with a B cell once the B cell has processed and presented a fragment of the antigen via its MHC (or HLA) class II molecules. T cells only recognize small linear peptides released from antigen and presented in the cleft of the class II molecule. The initial activation of CD4⁺ T cells occurs when a specialized antigen presentation cell, the dendritic cell, processes and presents peptide fragments to naïve T cells [4–6].

It is useful to think of allergy as a subset of immunity with two distinct phases: the sensitization phase where the initial CD4⁺ T cell activation occurs, triggering a process that drives the downstream events described above. The second phase is the more recognizable, distinctly 'allergic' phase, where an encounter with the protein immunogen triggers mediator release (e.g. histamine) from the previously IgE-loaded mast cells.

Th2 cells and IgE-mediated allergic responses

The immune response that must occur in order to precipitate an IgE-mediated type I allergic response requires CD4⁺ T cells capable of secreting IL-4 or IL-13 [7]. T cells capable of inducing an allergic response by means of their cytokine expression are called Th2 cells. These cells differentiate from uncommitted precursors, and understanding the signals that control the differentiation of Th2 as opposed to Th1 or T regulatory cells are an area of intense study and controversy. It is clear that

activation of CD4⁺ T cells in the presence of IL-4 will induce differentiation to Th2-type cells and that the presence of IL-12 will suppress Th2 development. However, a number of other factors are likely to contribute in a non-exclusive manner: (1) the state of maturation of the antigen-presenting dendritic cell; (2) the costimulatory molecules and receptors present, that may depend on the maturation state of the dendritic cell [8, 9]; (3) the level of antigen and the extent of TCR engagement [10]; (4) the type of adjuvant used, including proteolytic activity of the protein immunogen itself [11]; and (5) the microenvironment in which the activation event takes place [12–14]. Additional factors are likely to contribute to the development of food allergies where the allergen itself must be capable of withstanding the conditions of the digestive tract in order to induce an immune response in the gut [15].

The increasing prevalence of allergic disease

Over the past 30 years, the prevalence of allergic diseases has markedly increased. The increase seems to be independent of improved diagnosis. The cause is not likely to be the fixation of susceptible alleles in the population as this has occurred within about one generation, although genetic associations have been detected between allergy and the presence of certain disease-modifying alleles [16]. Factors associated with the increase in allergic disease include childhood vaccination and the concomitant loss of infections early in life, and the lack of exposure to bacterial endotoxin commonly associated with rural living conditions (the Hygiene Hypothesis [17–19]). Increasing predisposition to develop allergies could be due to either the establishment of a Th2-preferred microenvironment on the organismal level, or the loss of the induction of T regulatory cells primed to blunt allergic-type responses [18, 20].

The 'Hygiene Hypothesis' is based on correlative observations in human populations, where the testing of mechanisms is not possible. A molecular mechanism has been suggested by genetic analysis of mouse strains that display a marked bias towards Th1- or Th2-type antigen responses. The mouse homologue (TIM-1) for the human hepatitis A virus receptor (HAV receptor) was shown to be a genetic marker for predisposition for Th2-type responses [16], and mouse strains that are known to be prone to develop Th2-type responses carry *Tim 1* alleles that contain polymorphisms in contrast to the *Tim 1* genes in Th2-resistant mice. In humans, there is an inverse correlation between hepatitis A infection and the development of allergy, suggesting that signalling via the HAV receptor can influence an individual's predisposition to develop an allergic response during a subsequent immunological encounter with an allergen. Similar mechanisms may be revealed in the case of endotoxin exposure, where early exposure to endotoxins and signalling via

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Toll-Like receptors (TLRs) may influence the phenotype of subsequent Th responses.

The increasing prevalence of allergic disease indicates that there is an immediate need both to focus on the effective treatment of pre-existing allergies, and to prevent the introduction of new protein allergens by assessing the potential allergenic impact of any changes in the exposure rate of the population to either new or modified proteins. These issues define two distinct subsets of the general population: those who have encountered an immunogenic protein in a manner that has resulted in the establishment of an allergic-type immune response, and those who are immunologically naïve to the putative protein immunogen. Immunologic intervention in these two populations will be distinct: the pre-exposed individual will need treatment to either limit or obviate an established immune response, while the unexposed individual must be protected from becoming sensitized.

Identification of T cell epitopes in allergens: pre-existing allergy applications

Allergic responses are initiated and prolonged by CD4⁺ T cells. The only effective therapeutic intervention for allergy is specific immunotherapy (SIT), where an allergic individual is repeatedly injected with increasing doses of allergen extract. Over time, the individual becomes less reactive to the allergen. The mechanism is unclear, but may be due to CD4⁺ T cell tolerance induction or to immune deviation away from an allergic-type response. This therapy has been proven to be effective in many but not all individuals. Non-responsiveness may be due to a number of factors, one of which could be the dose-limiting toxicity of inducing a deleterious allergic response. In addition, allergen preparations are poorly standardized. The need for a rigorously controlled product that can be administered in a wider dose range has driven the development of the following therapies.

Peptide immunotherapy

Peptide immunotherapy reduces the allergen to its component CD4⁺ T cell epitopes. Peptides are administered in order to induce antigen-specific CD4⁺ T cell tolerance. Because the peptides cannot cross-link IgE, they represent a safer therapy for SIT than intact allergens [21]. This method has been shown to be effective in animal models, and there are some promising early results in humans [22, 23]. As this method relies on HLA binding peptides, it is important to verify the population-based immunodominant peptides for each ethnic group under consideration [24]. The mechanism of action of peptide immunotherapy remains unresolved but may include the induction of anergy [25, 26], or the induction of antigen-specific T regulatory cells mediating their effect via IL-10 [27].

Protein-based immunotherapy

Another method to improve the safety and efficacy of SIT is the construction of largely intact recombinant allergens modified to reduce IgE binding, but with all potential T cell epitopes intact.

Alternately, naturally occurring allergen variants can be selected which contain cross-reactive T cell epitopes but no IgE binding sites [28, 29]. Recombinant IgE binding site modified allergen molecules have been shown to induce tolerance in animal models [30, 31], and while the mechanism of tolerance induction is unknown, it may reflect the up-regulation of allergen-specific IgG, the activation of antigen-specific regulatory T cells, or perhaps via the recruitment of inappropriate antigen-presenting cells [32].

Methods for inducing allergen nonresponsiveness that involve peptides or IgE binding site-modified proteins require the identification of individual allergens for inclusion in a therapy regimen on a per patient basis. Most individuals with allergies are responsive to many different proteins, due to exposure to a mixture of proteins (such as those present in a pollen grain) or to the individual's predisposed nature. Either way, a cocktail approach would need to be considered for either of these two applications to become universally effective.

Both methods depend on the identification of T cell epitopes in the proteins of interest, either to excise them for use in peptide immunotherapy, or to avoid modifying them during the creation of recombinant IgE binding site-modified proteins.

Identification of T cell epitopes in allergens: prevention of sensitization

Protein therapeutics

The creation of recombinant proteins that do not initiate an immune reaction is of immediate concern to the development of protein therapeutics. Severe consequences of immune activation have been reported for protein therapeutics, for example pure red cell aplasia as a consequence of recombinant human erythropoietin administration [33]. This induction of autoantibodies to a normal human protein is a serious limitation of protein therapy, as the therapeutic cannot be 'discontinued'.

The bacterial protein staphylokinase represents another important example. This molecule is effective in dissolving blood clots during myocardial infarctions [34], yet its therapeutic use is limited by the molecule's ability to induce antigen-specific IgG that neutralizes the effectiveness of the therapy. The IgG binding sites in the molecule were defined and modified in an elegant series of experiments [35–40], but after testing human subjects with the sequence modified, PEG-ylated protein, the molecule was still deemed too immunogenic for repeat dosing. Recently, a number of CD4⁺ T cell epitopes have been identified in staphylokinase, and T cell epitope modified variants have been created that reduce proliferation by epitope-specific T cell clones [41, 42]. Finally, therapeutic antibodies derived from mice have a well-characterized propensity for inducing human anti-mouse antibodies. This has been addressed by 'humanizing' the antibodies, creating chimeric human/mouse molecules, or by constructing fully human antibodies [43–46]. All of these methods of reducing the immunogenicity of antibody therapeutics have shown some level of success [47, 48].

Transgenic foods

Transgenic foods hold the promise of increased nutrition, improved disease and pesticide resistance, and improved economics (shelf life, handling). However, the introduction of novel or

'rare' proteins into the food chain is a cause of concern. The appearance of a new protein in food, or an increase in the level of a food protein, may be a trigger for food allergy in a susceptible individual. For example, the 2S storage globulin found in Brazil nuts, a known food allergen [49], has been found to contain a number of prominent CD4⁺ T cell epitopes [50]. Brazil nuts are consumed frequently in the United States, although not nearly as often as other sensitizing nuts such as peanuts. Ber e 1, the 2S storage globulin in Brazil nut is a very nutritious protein. Based on this property it was incorporated into soybeans as a way to improve the food value. Unfortunately, the Ber e 1 product in transgenic soy was recognized by antibodies from serum of Brazil nut-allergic individuals [51]. This example serves as a reminder to the scientific community that the introduction of novel proteins, or the introduction of potentially allergenic proteins into sources where they are not anticipated could be problematic [52]. There has been focus on methods to identify potential food allergens, via sequence homology with known allergens, as well as their digestibility and acid-stability [15, 53, 54]. These methods are used to identify potential transgenes that encode proteins with significant sequence homology and antibody cross-reactivity to known food allergens that should not be developed in order to protect the pre-exposed segment of the population. However, for the introduction of unique proteins, modified proteins generally regarded as safe, or proteins that have not imprinted themselves on the immune system of the general public, the primary concern is to identify which of these proteins could induce *de novo* an inappropriate Th2-type of immune response, a response dependent on the presence of CD4⁺ T cell epitopes.

Methods for identifying T cell epitopes in proteins

Predictive methods

Three methods are used to predict T cell epitopes from the primary sequence of proteins: (1) Motif-based predictive algorithms based on peptide binding signatures for HLA alleles derived from experimental data (for example [55]); (2) statistical-derived methods including quantitative matrix and neural network modelling [56]; and (3) structure-based methods where binding of peptides to HLA are modelled using 3-dimensional crystallographic tools. Combinations of these methods are often used and have shown themselves to be quite accurate for the prediction of HLA class I-binding epitopes. In order to apply these methods to the selection of epitopes for more than an individual HLA allele, the motifs, algorithms or matrix data must be compiled. The computing power needed for analysis of the multiple HLA class II alleles commonly found in the human population can be prohibitive as there are many HLA class II alleles (Fig. 1). In addition, the rigorousness of these methods depends on the accuracy and availability of experimental data [57].

The binding of peptide epitopes to HLA molecules is defined by the specific sequence of the peptide and its structural complementarity to the configuration of the peptide-binding cleft. In contrast to HLA class I, HLA class II rules are difficult to rigorously define as there is significant degeneracy in the specificity of peptide binding to HLA-DR alleles, and particular peptide sequences can promiscuously bind to multiple HLA-DR alleles [58, 59]. To address this issue within the context of

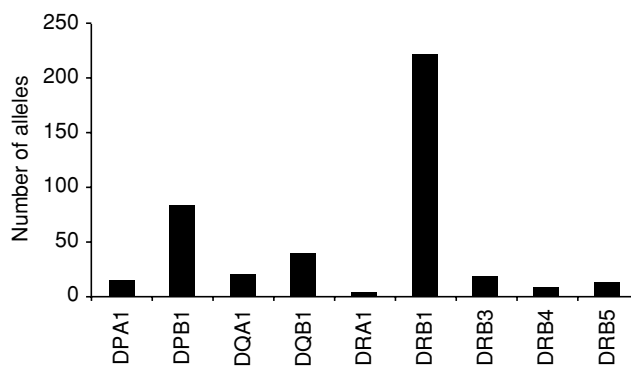


Fig. 1. HLA class II alleles. The alpha and beta chain polymorphisms for HLA-DP, HLA-DQ and HLA-DR are enumerated.

creating a predictive algorithm, Sturinolo et al. [60] analysed binding of peptides to multiple HLA-DR alleles and devised a method that predicts peptide binding based on the specificity of binding within a family of 35 pocket profiles which define peptide binding to (almost all) HLA-DR specificities. The resulting computer-based program, TEPITOPE, is widely utilized to predict promiscuously binding T cell epitopes in proteins for the development of vaccines [61–63].

The weakness of all computer-based methods lies in their reliance on HLA-DR. Other HLA class II molecules including HLA-DQ and HLA-DP proteins are expressed at lower levels on the surface of antigen-presenting cells [64] but CD4⁺ T cell responses to these proteins can be easily detected [65]. HLA-DQ is becoming an increasingly important molecule in the restriction of allergic and pathological responses [66, 67], yet computer algorithms contain little motif data. This is due to the demonstrated lack of specific binding motifs for some HLA-DQ molecules [68], their relatively high degree of promiscuity [69], and their capacity to form allelic heterodimers that create new peptide specificities. There exists even less motif or matrix data for HLA-DP, the most conserved class II molecule expressed [70].

Additional defects with predictive models include the following: binding to class II molecules is a good indicator of epitope status, but does not completely correlate with CD4⁺ T cell activation [71, 72]. Contribution of flanking residues to T cell activation has been demonstrated [73, 74], but is not accounted for in predictive models. Finally, all of the described predictive methods are useful for selecting subsets of the total number of peptides described by each protein sequence for subsequent *in vitro* screening. False negatives are clearly a significant problem when a protein contains prominent HLA-DQ or HLA-DP restricted epitopes, but they can also result from the presence of poorly binding yet immunogenic peptide epitopes. False positives occur often but can be removed from consideration by additional screening. False negatives, however, cannot be addressed unless the entire protein is screened. Additional *in vitro* screening usually consists of peptide binding (competition) assays with purified HLA molecules and *in vitro* proliferative assays using antigen-specific T cells. Antigen-specific T cells are obtained either from sensitized donors or are expanded from unexposed donors by multiple rounds of re-stimulation. Both of these methods of validating predicted epitopes have their inherent problems, but the lack of significant numbers of sensitized donors to the protein of interest is

potentially the most problematic issue. Testing peptides with cells from a sensitized donor reveals not only that a peptide represents an epitope recognized by memory T cells, but also shows that the particular epitope is processed and presented from the protein *in vivo*.

***In vitro* methods for epitope identification and validation**

The activation of individual CD4⁺ T helper cells is highly complex, involving the colocalization of a number of signalling molecules and adhesion molecules and their reorganization into an immune synapse. Downstream signalling complexity stems from the initial antigen-specific interaction and while the trimolecular complex of HLA, peptide and TCR is required for antigen-specific signalling to occur, a number of other variables including the quality of the synapse and the microenvironmental milieu control the outcome of cellular signalling. To be assured that a given peptide/HLA complex can induce signalling leading to a cellular activation event, *in vitro* validation with human CD4⁺ T cells is necessary.

The validation of predicted CD4⁺ T cell epitope peptides involves using cells from sensitized donors to determine whether a previously exposed donor has mounted a response to that particular peptide [75]. Most protocols involve testing synthetic peptides or fragments of the proteins of interest generated by enzymatic or chemical treatment. PBMC from sensitized human donors are cultured *in vitro* with the peptides or protein fragments, and a response is quantified by any number of means including proliferation or cytokine secretion. A limitation of this method is that it does not reveal whether the epitope peptide is capable of inducing a primary response in an unexposed donor. Epitope peptides recognized by memory and naïve CD4⁺ T cells can be distinct [76, 77] as has been shown for T cell clones isolated from multiple sclerosis patients bearing a response to myelin basic protein (MBP) [76]. CD4⁺ T cell clones responded to distinct peptides based on whether they were derived from CD45RA⁺ (naïve) or the CD45RO⁺ (memory) subset. This could be a consequence of 'epitope-spreading' which has been widely characterized in autoimmune diseases, and recently in cancer immunotherapy [78]. The mechanism of intramolecular epitope spreading is unknown, but the specificity-broadening effects of CTLA-4 on T cell immune responses [79, 80] have been shown to both positively and negatively affect epitope spreading in various mouse models of autoimmunity [81, 82].

The selection of food transgenes and the creation of hypoallergenic proteins requires the identification of a subset of CD4⁺ T cell epitopes capable of initiating an immune cascade resulting in diverse memory responses and epitope spreading. These represent a subset of the total number of epitope responses recorded when testing unseparated CD4⁺ T cells from sensitized donors *in vitro*. Thus proper determination of these epitopes requires testing donors unexposed to the protein in question. Further, to determine the dominant responses in a population a large diverse set of unexposed donors must be tested from which to define HLA-associated vs. promiscuous binding status.

Bearing in mind the limitations of predictive algorithms, the need to identify immunodominant epitopes on a population

basis, and in the absence of significant numbers of individuals presensitized to many proteins of interest, the *i*-mune[®] assay was developed [83].

The *i*-mune assay for epitope identification

The *i*-mune assay is conceptually similar to methods described for validating predicted epitopes using sensitized donors [83] (Fig. 2). However, critical differences make the *i*-mune assay unique. First, community donors selected randomly from local blood donation centres provide the source of PBMC. Blood donors in the United States are pre-selected using a self-reporting system that asks the health status of each individual. Donors who are at high risk for being exposed to hepatitis viruses, HIV or CJD-associated prions are excluded, and the genetics of the population is not statistically different from the United States general population. This was verified by an analysis of HLA-DR alleles in the donor population. Second, dendritic cells are generated from each donor to use as autologous antigen-presenting cells. Dendritic cells are the most potent antigen-presenting cell type described, and are the only cells capable of activating resting, naïve CD4⁺ T cells [6, 84]. In addition, the dendritic cells generated are assayed in serum-free conditions, which reduces the level of background responses and focuses the response on exogenously added peptides. Third, CD4⁺ T cells are enriched from the autologous donor for inclusion in the assay in order to increase the probability of assaying naïve CD4⁺ T cells that may be present at very low precursor frequencies. Finally, arrays of peptides are assayed in large donor sets to verify the immunodominance of particular epitope peptide responses.

i-mune assay results are dependent on the immunological status of the donor population. For example, where the donor population is unexposed to the protein of interest, the *i*-mune assay localizes immunodominant epitopes for the naïve CD4⁺ T cell population as exemplified by the *i*-mune results for industrial enzymes such as *B. lentus* subtilisin where the general population is not commonly exposed. The donor pool appears to be 'unexposed' to human-derived proteins that are not ubiquitously expressed and therefore do not induce central or peripheral tolerance. Alternatively, when a significant number of donors in the pool have been pre-exposed to the protein of interest, the *i*-mune assay will identify promiscuous and HLA-associated memory and naïve epitopes. For most of the common allergens, our donors represent both allergic and non-allergic exposed donors. Interestingly, both allergic and non-allergic exposed donors recognize the same T cell epitopes [84]. The immunodominant priming epitope will be present in the set of identified epitopes, but it may be indistinguishable from the memory epitopes (see below).

Epitope identification: memory and primary responses

To test the predictive value of the *i*-mune assay, *i*-mune data generated using community donors was compared to epitopes described in verified protein-exposed donors (Fig. 3). Data for two model proteins are shown. Responses to staphylokinase accumulate in the population with age [41] so the donor population tested likely contained individuals with memory

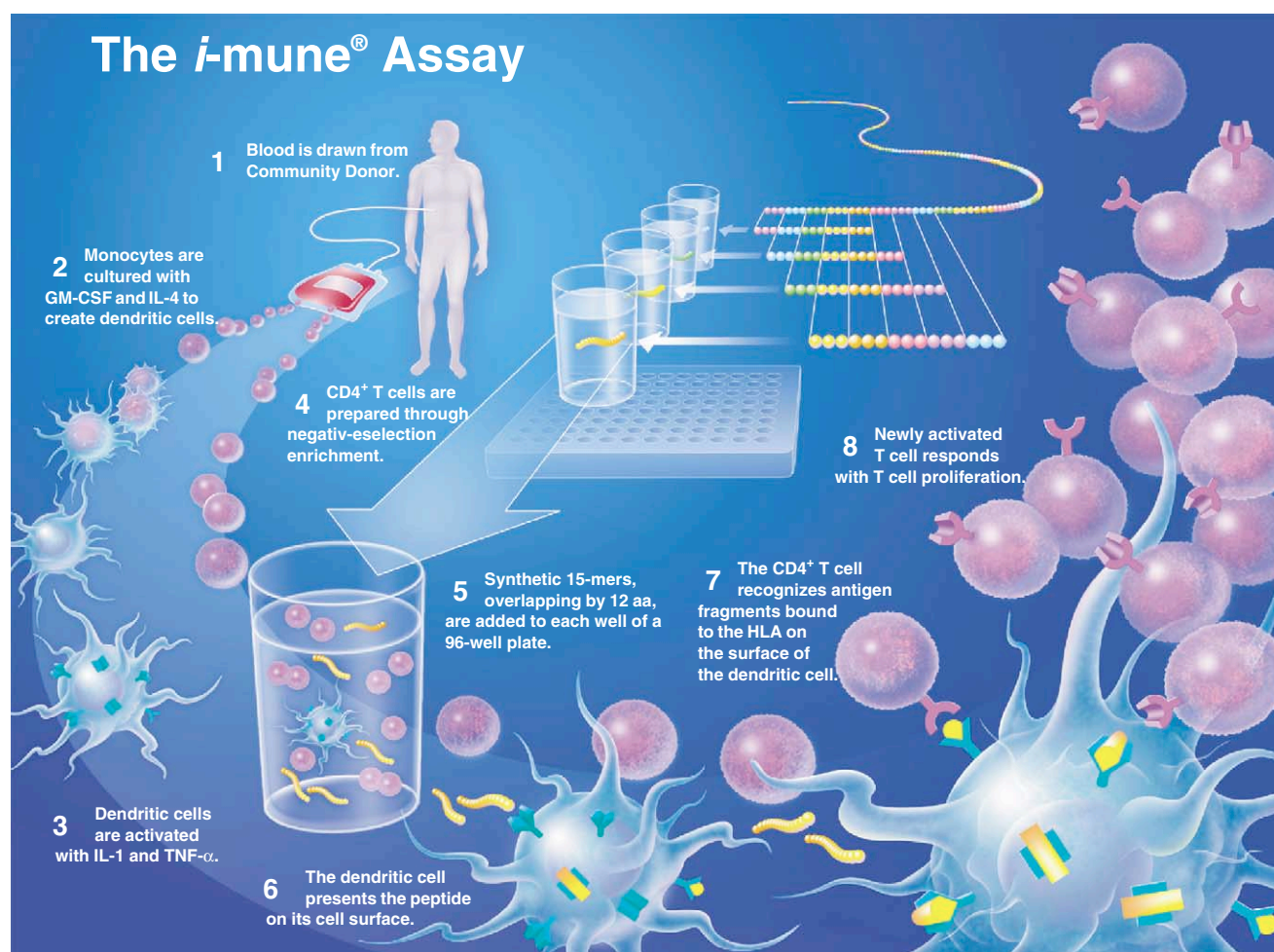


Fig. 2. The *i*-mune assay.

responses to this protein. The industrial enzyme *B. lentus* subtilisin is rarely encountered outside of industrial settings, and the general population is not detectably exposed (as an example, [86]). To assay these proteins, synthetic 15-mer peptides offset by three amino acids describing the entire sequence of the two proteins were synthesized using Multi-Pin technology [87].

Memory responses in the donor pool

A total of 72 donors were tested with staphylokinase peptides. Each donor's response was tabulated. Five peptides induced responses in a statistically significant number of donors: amino acids 13-27, 58-75, 85-99 and 106-120. Statistical significance was determined by defining a Poisson distribution around the average background value, then testing the prominent epitope responses for difference from the background. The background value was 6.22%. All epitope peptides described were different from the background ($P < 0.05$). Two separate epitope peptides were defined in the amino acids 58-75 region. These data were concordant with published information [41,42]. Six largely overlapping epitope regions were described for

staphylokinase using T cell clones isolated from PBMC of 10 healthy human donors. The *i*-mune assay localized epitope peptides corresponding to all of the general regions (Fig. 3a). The *i*-mune assay also localized an epitope peptide, amino acids 85-99, which was not detected using cloned human cells. This peptide may not be processed from the protein, or may have been missed in the cloned cell analysis due to the low number of individuals tested. In fact, a significant association between HLA-DR11(5) and this peptide was found, and only one HLA-DR11(5)-carrying donor was tested in the published study. In agreement with the published results, the responses to most of the epitope peptides in the community donor data were promiscuous, with the exception of peptide 58-75 where an association with HLA-DR15(2) was evident.

Primary responses by the donor pool

A total of 65 community donors were tested with peptides synthesized corresponding to the sequence of *B. lentus* subtilisin. This data was expanded from that reported in [83]. The background response to this protein was much lower than the background level found with staphylokinase: 3.45%, and only

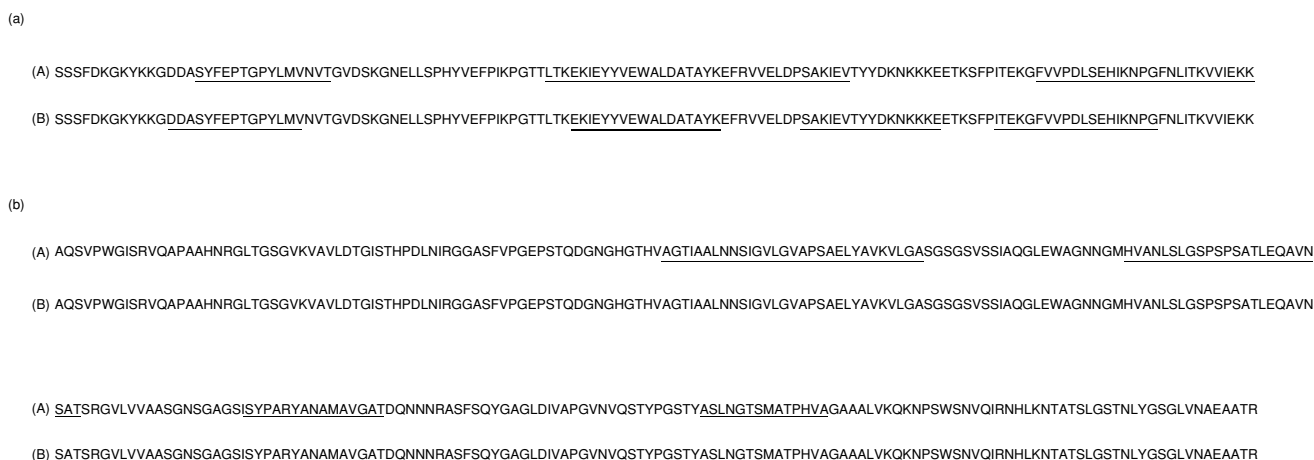


Fig. 3. (a) CD4⁺ T cell epitopes identified by antigen-specific clones (A), or by the *i*-mune[®] assay (B), in Staphylokinase (SakSTAR). A. CD4⁺ T cell epitopes in SakSTAR are reproduced from data presented by Warmerdam, et al. [41]. B. The *i*-mune[®] assay was performed on 72 community donors. The underlined epitope regions represent peptides that induced responses at a frequency that was statistically different ($P < 0.05$) from the background rate. The background rate was calculated by averaging the responses to every peptide for the complete dataset. (b) CD4⁺ T cell epitopes identified using PBMC from seven *B. lentus* subtilisin SPT⁺ donors (A), or by the *i*-mune[®] assay (B). Line A is the continuous amino acid sequence of *B. lentus* subtilisin (total of 269 amino acids). Line B is the continuous sequence of *B. lentus* subtilisin. A. Seven *B. lentus* subtilisin SPT⁺ donors were tested for peptide-specific responses in the *i*-mune[®] assay. Underlined peptide sequences indicate where two or more donors responded with a stimulation index of greater than or equal to 2.95. B. The *i*-mune[®] assay was performed on 65 community donors. The underlined epitope region represents the peptide that induced a response at a frequency that was statistically different ($P < 0.001$) from the background rate. Only one epitope was found. The background rate was calculated by averaging the responses to every peptide for the complete dataset.

one significant epitope was found in *B. lentus* subtilisin at amino acids number 160–174. In order to verify this result, a total of seven SPT⁺ donors were tested in the *i*-mune assay. The results are shown in Fig. 3(b). Peptides that elicited proliferative responses with a stimulation index of 2.95 or greater in two or more of the seven donors are indicated. The single epitope recognized by the community donors is recognized by three of the seven SPT⁺ donors. All other underlined regions elicited responses in two of seven donors. The epitope response in the community donors was restricted to HLA-DR15(2). Interestingly, both of the HLA-DR15(2) donors in the SPT⁺ group also responded to this epitope.

In the *i*-mune assay, staphylokinase demonstrates the behaviour of a protein that has induced a memory response in some donors in that the overall background level responses to peptides in this set are higher than they are for rarely encountered industrial proteins. A pre-existing memory response to staphylokinase accounts for finding the same epitope regions characterized using the T cell clones. In contrast, the result with *B. lentus* subtilisin peptides, derived from a protein not routinely encountered by the donor population and where memory responses are rare, was that a subset of the epitopes is detected. The subset of epitopes described when using presumably unexposed donors represents peptide epitopes likely to be capable of inducing a primary response, and are therefore described as immunodominant for the population tested.

i-mune assay: creation of reduced immunogenicity protein variants

The *i*-mune assay has been applied to the design of reduced immunogenicity protein moieties. In all cases, the biological functions of the molecules remained intact. Single point mutations were made in the identified epitopes of the proteins, and the expressed molecules were tested in animal models. Animal

models were selected for their correspondence with the human cell-based results. A number of mouse strains, both common laboratory strains and human HLA class II transgenic strains, were screened for epitope responses that aligned with the results from the *i*-mune assay. In the selected mouse models, molecules that were modified based on *i*-mune results were found to induce less T cell proliferation and less IgG1 antibody than the parent molecules. For a modified protease, a 2–5-fold increase in the amount of protein needed to induce 50% maximum response was measured, with a 10-fold reduction in peak IgG1 titres.

In addition to animal models, modified proteins were tested for their ability to induce proliferation in cultures of unseparated human PBMC [41]. The modified protease described above induced a reduced frequency and level of proliferation in these cultures that was statistically significant (parametric two-tailed Student's *t*-test $P = 0.007$).

Summary

The identification of T cell epitopes is a critical component of the design and implementation of therapies for pre-existing allergy. The identification of T cell epitopes in potential protein therapeutics and food transgenes can serve to guide the selection or creation of hypo-immunogenic candidates. Computer-based methods are useful, but have serious deficiencies, the most profound being a lack of modelling for HLA-DQ and HLA-DP. All computer-predicted epitopes must be validated *in vitro* by subsequent binding and activation assays. The *i*-mune assay represents an alternative to computer-based models that entails no *a priori* selection for HLA molecules via either a lack of predictive algorithms or a lack of significant numbers of donors, limits the risk of false negative results, and provides a wealth of functional information for any protein of interest.

References

- 1 Stavnezer J. Immunoglobulin class switching. *Curr Opin Immunol* 1996; 8:199–205.
- 2 Punnonen J, Aversa G, Cocks BG et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993; 90:3730–4.
- 3 Grusby M, Johnson R, Papaioannou V, Glimcher L. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. *Science* 1991; 253:1417–20.
- 4 Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell* 2001; 106:263–6.
- 5 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; 9:271–96.
- 6 Viola A, Iezzi G, Lanzavecchia A. The role of dendritic cells in T cell priming: the importance of being professional. In: Lotze MT, Thomson AW, eds. *Dendritic Cells: Biology, Clinical Applications*. San Diego: Academic Press, 1999;251–5.
- 7 Mazzarella G, Bianco A, Catena E, De Palma R, Abbate GF. Th1/Th2 lymphocyte polarization in asthma. *Allergy* 2000; 55 (Suppl. 61):6–9.
- 8 Oosterwegel MA, Mandelbrot DA, Boyd SD et al. The role of CTLA-4 in regulating Th2 differentiation. *J Immunol* 1999; 163:2634–9.
- 9 Kuchroo VK, Das MP, Brown JA et al. B7–1 and B7–2 Costimulatory Molecules Activate Differentially the Th1/Th2 Developmental Pathways: Application to Autoimmune Disease Therapy. *Cell* 1995; 80:707–18.
- 10 Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med* 1995; 182:1591–6.
- 11 Hewitt CR, Foster S, Phillips C et al. Mite allergens: significance of enzymatic activity. *Allergy* 1998; 53:60–3.
- 12 Constant SL, Lee KS, Bottomly K. Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential Th2-type differentiation. *Eur J Immunol* 2000; 30:840–7.
- 13 Stumbles PA, Thomas JA, Pimm CL et al. Resting respiratory tract dendritic cells preferentially stimulate T helper type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. *J Exp Med* 1998; 188:2019–1031.
- 14 Constant SL, Bottomly K. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* 1997; 15:297–322.
- 15 Lehrer SB, Ayuso R, Reese G. Current understanding of food allergens. *Ann NY Acad Sci* 2002; 964:69–85.
- 16 McIntire JJ, Umetsu SE, Akbari O et al. Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. *Nat Immunol* 2001; 2:1109–16.
- 17 Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH et al. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002; 3:715–20.
- 18 Braun-Fahrlander C, Riedler J, Herz U et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002; 347:869–77.
- 19 Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002; 347:911–20.
- 20 Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat Rev Immunol* 2001; 1:69–75.
- 21 Ali FR, Kay AB, Larche M. The potential of peptide immunotherapy in allergy and asthma. *Curr Allergy Asthma Rep* 2002; 2:151–8.
- 22 Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002; 360:47–53.
- 23 Oldfield WL, Kay AB, Larche M. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. *J Immunol* 2001; 167:1734–9.
- 24 Inoue R, Matsushita S, Kaneko H et al. Identification of beta-lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy. *Clin Exp Allergy* 2001; 31:1126–34.
- 25 Gaur A, Wiers B, Liu A, Rothbard J, Fathman CG. Amelioration of Autoimmune Encephalomyelitis by Myelin Basic Protein Synthetic Peptide-Induced Anergy. *Science* 1992; 258:1491–4.
- 26 Briner TJ, Kuo M-C, Keating KM, Rogers BL, Greenstein JL. Peripheral T-cell Tolerance Induced in Naive and Primed Mice by Subcutaneous Injection of Peptides from the Major Cat Allergen Fel d 1. *Proceeding Natl Academy Sci, USA* 1993; 90:7608–12.
- 27 Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102:98–106.
- 28 Ferreira F, Wallner M, Breiteneder H, Hartl A, Thalhamer J, Ebner C. Genetic engineering of allergens: future therapeutic products. *Int Arch Allergy Immunol* 2002; 128:171–8.
- 29 Eriksson TL, Gafvelin G, Elfman LH, Johansson C, Van Hage-Hamsten M, Olsson S. T cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*. *Clin Exp Allergy* 2001; 31:1881–90.
- 30 Wiedermann U, Herz U, Vrtala S et al. Mucosal tolerance induction with hypoallergenic molecules in a murine model of allergic asthma. *Int Arch Allergy Immunol* 2001; 124:391–4.
- 31 Wiedermann U, Herz U, Baier K et al. Intranasal treatment with a recombinant hypoallergenic derivative of the major birch pollen allergen Bet v 1 prevents allergic sensitization and airway inflammation in mice. *International Arch Allergy Immunol* 2001; 126:68–77.
- 32 Tsitoura DC, Yeung VP, DeKruyff RH, Umetsu DT. Critical role of B cells in the development of T cell tolerance to aeroallergens. *Int Immunol* 2002; 14:659–67.
- 33 Casadevall N, Nataf J, Viron B et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med* 2002; 346:469–75.
- 34 Vanderschueren S, Barrios L, Kerdsinchai P et al. A randomized trial of recombinant staphylokinase versus alteplase for coronary artery patency in acute myocardial infarction. The STAR Trial Group. *Circulation* 1995; 92:2044–9.
- 35 Collen D, Bernaerts R, Declercq P et al. Recombinant staphylokinase variants with altered immunoreactivity. I. Construction and characterization. *Circulation* 1996; 94:197–206.
- 36 Collen D, Moreau H, Stockx L, Vanderschueren S. Recombinant staphylokinase variants with altered immunoreactivity. II. Thrombolytic properties and antibody induction. *Circulation* 1996; 94:207–16.
- 37 Collen D, Stockx L, Lacroix H, Suy R, Vanderschueren S. Recombinant staphylokinase variants with altered immunoreactivity. IV. Identification of variants with reduced antibody induction but intact potency. *Circulation* 1997; 95:463–72.
- 38 Collen D, De Cock F, Demarsin E et al. Recombinant staphylokinase variants with altered immunoreactivity. III. Species variability of antibody binding patterns. *Circulation* 1997; 95:455–62.
- 39 Collen D, Sinnaeve P, Demarsin E et al. Polyethylene glycol-derivatized cysteine-substitution variants of recombinant staphylokinase for single-bolus treatment of acute myocardial infarction. *Circulation* 2000; 102:1766–72.
- 40 Laroche Y, Heymans S, Capaert S, De Cock F, Demarsin E, Collen D. Recombinant staphylokinase variants with reduced

- antigenicity due to elimination of B-lymphocyte epitopes. *Blood* 2000; 96:1425–32.
- 41 Warmerdam PA, Vanderlick K, Vandervoort P et al. Staphylokinase-specific cell-mediated immunity in humans. *J Immunol* 2002; 168:155–61.
 - 42 Warmerdam PA, Plaisance S, Vanderlick K et al. Elimination of a human T-cell region in staphylokinase by T-cell screening and computer modeling. *Thromb Haemost* 2002; 87:666–73.
 - 43 Kashmiri SV, Iwahashi M, Tamura M, Padlan EA, Milenic DE, Schlom J. Development of a minimally immunogenic variant of humanized anti-carcinoma monoclonal antibody CC49. *Crit Rev Oncol Hematol* 2001; 38:3–16.
 - 44 Merluzzi S, Figini M, Colombatti A, Canevari S, Pucillo C. Humanized antibodies as potential drugs for therapeutic use. *Adv Clin Path* 2000; 4:77–85.
 - 45 Lonberg N, Taylor LD, Harding FA et al. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* 1994; 368:856–9.
 - 46 Fishwild DM, O'Donnell SL, Bengoechea T et al. High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat Biotechnol* 1996; 14:845–51.
 - 47 Mountain A, Adair JR. Engineering antibodies for therapy. *Biotechnol Genet Eng Rev* 1992; 10:1–142.
 - 48 Klingbeil C, Hsu DH. Pharmacology and safety assessment of humanized monoclonal antibodies for therapeutic use. *Toxicol Pathol* 1999; 27:1–3.
 - 49 Sicherer SH, Sampson HA. Peanut and tree nut allergy. *Curr Opin Pediatr* 2000; 12:567–73.
 - 50 Stickler MM, Mucha J, Estell D, Power S, Harding FA. A human dendritic cell-based method to identify CD4(+) T-cell epitopes in potential protein allergens. *J Env Health Persp* 2003; 111:251–4.
 - 51 Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RK et al. Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med* 1996; 334:688–92.
 - 52 Lack G. Clinical risk assessment of GM foods. *Toxicol Lett* 2002; 127:337–40.
 - 53 Oehlschlager S, Reece P, Brown A et al. Food allergy—towards predictive testing for novel foods. *Food Addit Contam* 2001; 18:1099–107.
 - 54 Hileman RE, Silvanovich A, Goodman RE et al. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. *Int Arch Allergy Immunol* 2002; 128:280–91.
 - 55 Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI database for MHC ligands and peptide motifs. *Immunogenetics* 1999; 50:213–9.
 - 56 Doytchinova IA, Flower DR. Quantitative approaches to computational vaccinology. *Immunol Cell Biol* 2002; 80:270–9.
 - 57 Yu K, Petrovsky N, Schonbach C, Koh JY, Brusica V. Methods for prediction of Peptide binding to MHC molecules: a comparative study. *Mol Med* 2002; 8:137–48.
 - 58 Ou D, Mitchell LA, Tingle AJ. HLA-DR restrictive supertypes dominate promiscuous T cell recognition: association of multiple HLA-DR molecules with susceptibility to autoimmune diseases. *J Rheumatol* 1997; 24:253–61.
 - 59 Doherty DG, Penzotti JE, Koelle DM et al. Structural basis of specificity and degeneracy of T cell recognition: pluriallelic restriction of T cell responses to a peptide antigen involves both specific and promiscuous interactions between the T cell receptor, peptide, and HLA-DR. *J Immunol* 1998; 161:3527–35.
 - 60 Sturniolo T, Bono E, Ding J et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nature Biotechnol* 1999; 17:555–61.
 - 61 Consogno G, Manici S, Facchinetti et al. Identification of immunodominant regions among promiscuous HLA-DR restricted CD4+ T cell epitopes on the tumor antigen MAGE-3. *Blood* 2003; 101:1038–44.
 - 62 Manici S, Sturniolo T, Imro MA et al. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11 [see comments]. *J Exp Med* 1999; 189:871–6.
 - 63 de Lalla C, Sturniolo T, Abbruzzese L et al. Cutting edge: identification of novel T cell epitopes in Lol p5a by computational prediction. *J Immunol* 1999; 163:1725–9.
 - 64 Alonso MC, Navarrete C, Solana R, Torres A, Pena J, Festenstein H et al. Differential expression of HLA-DR and HLA-DQ antigens on normal cells of the myelomonocytic lineage. *Tissue Antigens* 1985; 26:310–7.
 - 65 Mellins E, Woelfel M, Pious D. Importance of HLA-DQ and –DP restriction elements in T-cell responses to soluble antigens: mutational analysis. *Hum Immunol* 1987; 18:211–23.
 - 66 Shan L et al. Structural basis for gluten intolerance in celiac sprue. *Science* 2002; 297:2275–9.
 - 67 Sonderstrup G, McDevitt HO. DR, DQ, and you: MHC alleles and autoimmunity. *J Clin Invest* 2001; 107:795–6.
 - 68 Raddizzani L, Sturniolo T, Guenot J et al. Different modes of peptide interaction enable HLA-DQ and HLA-DR molecules to bind diverse peptide repertoires. *J Immunol* 1997; 159:703–11.
 - 69 Sidney J, Del Guercio MF, Southwood S, Sette A. The HLA Molecules DQA1*0501/B1*0201 and DQA1*0301/B1*0302 Share an Extensive Overlap in Peptide Binding Specificity. *J Immunol* 2002; 169:5098–108.
 - 70 al-Daccak R, Wang FQ, Theophile D, Lethielleux P, Colombani J, Loiseau P. Gene polymorphism of HLA-DPB1 and DPA1 loci in caucasoid population: frequencies and DPB1–DPA1 associations. *Hum Immunol* 1991; 31:277–85.
 - 71 Velazquez C, DiPaolo R, Unanue ER. Quantitation of lysozyme peptides bound to class II mhc molecules indicates very large differences in levels of presentation. *J Immunol* 2001; 166:5488–94.
 - 72 DiPaolo RJ, Unanue ER. Cutting edge: chemical dominance does not relate to immunodominance: studies of the CD4+ T cell response to a model antigen. *J Immunol* 2002; 169:1–4.
 - 73 Carson RT, Vignali KM, Woodland DL, Vignali DAA. T Cell receptor recognition of MHC Class II-bound peptide flanking residues enhances immunogenicity and results in altered TCR V region usage. *Immunity* 1997; 7:387–99.
 - 74 Vignali DA, Strominger JL. Amino acid residues that flank core peptide epitopes and the extracellular domains of CD4 modulate differential signaling through the T cell receptor. *J Exp Med* 1994; 179:1945–56.
 - 75 Reece JC, McGregor DL, Geysen HM, Rodda SJ. Scanning for T helper epitopes with human PBMC using pools of short synthetic peptides. *J Immunol Meth* 1994; 172:241–54.
 - 76 Muraro PA, Pette M, Bielekova B, McFarland HF, Martin R. Human autoreactive CD4+ T cells from naive CD45RA+ and memory CD45RO+ subsets differ with respect to epitope specificity and functional antigen avidity. *J Immunol* 2000; 164:5474–81.
 - 77 Ramage JM, Young JL, Goodall JC, Gaston JSH. T cell responses to heat-shock protein 60: differential responses by CD4+ T cell subsets according to their expression of CD45 isotypes. *J Immunol* 1999; 162:704–10.
 - 78 Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* 2002; 2:85–95.
 - 79 Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol* 2001; 19:565–94.
 - 80 Kuhns MS, Epshteyn V, Sobel RA, Allison JP. Cytotoxic T lymphocyte antigen-4 (CTLA-4) regulates the size, reactivity, and function of a primed pool of CD4+ T cells. *Proc Natl Acad Sci USA* 2000; 97:12711–6.
 - 81 Wang HB, Shi FD, Li H, Chambers BJ, Link H, Ljunggren HG. Anti-CTLA-4 antibody treatment triggers determinant spreading

- and enhances murine myasthenia gravis. *J Immunol* 2001; 166:6430–6.
- 82 Karandikar NJ, Eagar TN, Vanderlugt CL, Bluestone JA, Miller SD. CTLA-4 downregulates epitope spreading and mediates remission in relapsing experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2000; 109:173–80.
- 83 Stickler MM, Estell DA, Harding FA. CD4+ T-cell epitope determination using unexposed human donor peripheral blood mononuclear cells. *J Immunother* 2000; 23:654–60.
- 84 Iezzi G, Karjalainen K, Lanzavecchia A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 1998; 8:89–95.
- 85 Carballido JM, Carballido-Perrig N, Kagi MK et al. T cell epitope specificity in human allergic and nonallergic subjects to bee venom phospholipase A2. *J Immunol* 1993; 150:3582–91.
- 86 Pepys J, Mitchell J, Hawkins R, Malo JL et al. A longitudinal study of possible allergy to enzyme detergents. *Clin Allergy* 1985; 15:101–15.
- 87 Maeji NJ, Bray AM, Geysen HM. Multi-pin peptide synthesis strategy for T cell determinant analysis. *J Immunol Meth* 1990; 134:23–33.