New Strategies for Allergen T Cell Epitope Identification: Going beyond IgE

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Key Words

Subcutaneous specific immunotherapy · T cell epitopes · Th2 cells · Timothy pollen

Abstract

Background: Type I allergy and allergic asthma are common diseases in the developed world associated with IgE antibodies and Th2 cell reactivity. To date, the only causative treatment for allergic disease is specific immunotherapy (SIT). **Method:** Here, we review recent works from our laboratory focused on identifying human T cell epitopes associated with allergic disease and their potential use as biomarkers or therapeutic targets for SIT. In previous studies, we have mapped T cell epitopes associated with the major 10 timothy grass (Tg) allergens, defined on the basis of human IgE reactivity by ELISPOT. **Results:** Interestingly, in about 33% of allergic donors, no T cell epitopes from overlapping peptides spanning the entire sequences of these allergens were identified despite vigorous T cell responses to the Tg extract. Using a bioinformatic-proteomic approach, we identified a set of 93 novel Tg proteins, many of which were found to elicit IL-5 production in T cells from allergic donors despite lacking IgE reactivity. Next, we assessed T cell responses to the novel Tg proteins in donors who had been treated with subcutaneous SIT. A subset of these proteins showed a strong reduction of IL-5 responses in donors who had received subcutaneous SIT compared to allergic donors, which correlated with patients' self-reported improvement of allergic symptoms. **Conclusion:** A bioinformatic-proteomic approach has successfully identified additional Tg-

derived T cell targets independent of IgE reactivity. This method can be applied to other allergies potentially leading to the discovery of promising therapeutic targets for allergen-specific immunotherapy.

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Introduction

In this review, we present an overview of present and historical work in our laboratory to identify and characterize T cell-stimulatory epitopes from known and previously undescribed timothy grass (Tg) proteins. Despite the importance of T cells in mediating type I allergy, there is still a significant lack of information on the epitopes they recognize. We combined several different methods with the intent to develop an approach for comprehensive T cell epitope mapping. As reviewed herein, this strategy is highly effective for mapping a highly diverse repertoire of T cell epitopes.

Allergic Disease in Modern Society

Allergic rhinoconjunctivitis is a common disorder in the developed world, posing a significant burden to the individuals who are directly affected, but also to society as a whole [1]. In a large scale study set out to measure the prevalence of allergic rhinitis among adults in Central Europe, it was reported that about 23% of the population suffered from clinically confirmable allergic rhinitis [2].

Similar data were obtained in studies conducted in children living in North America, estimating that approximately 13–17% of children in the United States suffer from allergic rhinitis [3, 4]. The clinical presentation includes nasal, ocular and throat symptoms associated with fatigue and other mood and cognitive disturbances [5]. Physical impairments and decreased quality of life are often underestimated and can be severe in both adults and adolescents. Moreover, type I allergy is frequently associated with asthma, a disease characterized by episodic exacerbations of partially reversible airflow limitations, bronchial hyperreactivity and airway inflammation [6]. Accordingly, significant effort has been made over the last decades to gain a better understanding of the causes and immunological events involved in this disease.

One of the most frequent triggers of allergenic rhinitis and asthma is grass pollen; irrespective of the latitude, it is found almost all over the world [7]. This trigger is estimated to be responsible for allergic symptoms in up to 50% of patients with allergy [8–10]. The resulting clinical manifestations range from milder symptoms such as rhinoconjunctivitis to severe asthma attacks [2]. Due to this high impact and clinical relevance, grass pollen allergy is among those most heavily studied. Tg represents one of the most common sources of grass pollen allergens in the world. In previous studies, 10 different Tg allergens have been identified based on their ability to bind to human IgE [11]. Over the past few decades, most of these allergens have been produced in recombinant form [12, 13]. IgE responses in Tg-allergic patients have been characterized [14], and many B and T cell epitopes have been identified [15–29]. This thorough characterization of the Tg-specific B and T cell repertoire in different donor cohorts makes Tg one of the most-well-studied allergenic triggers to date in terms of immune response targets.

Identification of T Cell Epitopes from Known and Novel Tg Antigens

The importance of T cells in the regulation and maintenance of allergic disease has been well established in the last decades. However, antigens that are considered allergenic triggers are typically defined based on their ability to bind specific IgE antibodies [11, 30] and induce IgE-mediated immediate hypersensitivity reactions [31], while the potential of the allergen to trigger T cell reactivity is in many cases not very well studied and is not taken into account when categorizing a protein as an allergen.

In a previous study, we performed a comprehensive screen to identify T cell epitopes from all 10 Phl p allergens known at the time [15]. Tg extract-stimulated peripheral blood mononuclear cells from allergic donors were cultured for 14 days with IL-2 and subsequently tested for T cell reactivity in response to a panel of overlapping peptides from all 10 allergens by means of IL-5 ELISPOT. During this screen, an interesting observation was made: While most of the donors responded to a variable selection of the peptides tested, in about a third of the donors no T cell response could be detected despite robust IL-5 production to Tg extract.

This observation led to the hypothesis that in addition to those allergens already identified by IgE reactivity, additional Tg proteins may be targeted by T cell responses. To test this hypothesis, we aimed to investigate T cell antigenicity of additional proteins on a broad scale. Since the Tg genome has not been sequenced, discovery of new Tg proteins required a sophisticated approach involving transcriptomic analysis of mRNA sequences obtained from Tg pollen coupled with a proteomic analysis to identify pollen proteins by mass spectrometry (MS) [32] (fig. 1). As a first step, Tg extract was run on a 2D gel, blotted onto nitrocellulose and probed with a serum pool from allergic patients to reveal immune reactivity of extract proteins. Although many of the extract proteins did bind IgE, a large fraction of proteins either only bound to IgG or were not recognized at all by antibodies despite being highly abundant in the Tg extract as revealed by the Coomassie stain. Several of these protein spots were cut out and subjected to MALDI-TOF MS analysis. Spectra obtained by MS were then compared to a cDNA library generated from mRNA sequences of Tg pollen, to reveal the transcripts that encoded the proteins.

This approach led to the identification of 93 novel Tg proteins [32]. As a next step, to test our hypothesis regarding the existence of additional Tg T cell antigens, we assessed whether any of these novel Tg proteins could induce T cell reactivity in cells from allergic donors. An HLA class II binding prediction algorithm previously validated [33] was employed to generate a library of candidate proteins. This allowed us to efficiently test for T cell reactivity, and bypass the need to express and isolate all 93 proteins or synthesize and test overlapping peptides for all identified proteins.

Accordingly, binding capacities of peptides derived from the novel Tg proteins were predicted for a panel of the 25 most common HLA MHC class II DR, DP and DQ molecules (fig. 1). This returned a set of 822 peptides predicted to bind promiscuously to MHC class II molecules

Fig. 1. A schematic presentation of the processes involved in the identification of novel Tg antigens and derived T cell epitopes. PBMC = Peripheral blood mononuclear cells.

(≥ 12 HLA variants predicted to be bound). These peptides were synthesized and tested as pools (~20 peptides per pool) in ELISPOT assays using Tg extract-expanded T cell cultures derived from 20 allergic donors (fig. 1). Twenty healthy donors were included as controls. Pools that elicited robust IL-5 or IFN γ responses were deconvoluted to identify the exact T cell epitope (fig. 1).

The data obtained from this screen clearly indicated that a significant fraction of the Th2 responses in allergic donors target T cell epitopes derived from these novel proteins. In fact, 13 out of 93 proteins elicited IL-5 responses in 20% or more of allergic donors tested [32]. In contrast, none of the antigens elicited IL-5 responses in more than 10% of control donors and the total response magnitude was 4-fold lower in controls compared to allergic donors. Table 1 shows a summary of these 13 antigens, their immunological reactivity and their identity as far as could be determined.

Interestingly, 7 of the 13 T cell antigens that elicited IL-5 in allergic donors did not exhibit any IgE binding as determined by the 2D immunoblot. Although this observation is very interesting, 2D immunoblot analysis is a crude method and not sufficiently sensitive for reliable characterization of antibody reactivity. The lack of IgE reactivity observed for the 7 antigens of interest will have to be confirmed in individual patients by additional methods such as radioallergosorbent test or immune solid-phase allergy chip.

Further characterization of the T cell responses targeting novel Tg antigens was performed to exclude artifacts induced by in vitro priming in the culture. Memory and naïve T cell populations were sorted ex vivo based on the expression of the chemokine markers CD45RA and CCR7. The sorted subsets were expanded in vitro with Tg extract. ELISPOT assays with pools from the most dominant IL-5-inducing peptides from either novel (19 peptides ac-

Table 1. Previously undescribed Tg antigens that elicit Th2 responses in ≥20% of patients tested

	Antigen	Antibody reactivity		T cell responses (20 donors)		Biochemical characteristics		
		IgE	IgG	% responders ¹	positive peptides ²	MW Da	rice homolog	biol. function
M09	49	–	–	25	24/26	61,860	putative L-ascorbate oxidase	multicopper oxidase
M34	54	–	–	20	8/12	61,860	putative L-ascorbate oxidase	multicopper oxidase
M76	91	–	–	20	5/5	19,987	hypothetical protein	cyclophilin-type peptidylprolyl <i>cis-trans</i> isomerases
M82	89	–	–	45	10/13	91,646	NA	NA
M83	22	–	–	20	3/5	29,529	NA	NA
MN26	53	–	+	30	4/5	33,002	glycerophosphoryl diester phosphodiesterase 2 precursor-like protein	glycerophosphodiester phosphodiesterase
M28	52	–	+	30	26/29	39,991	NA	NA
MN23	24	+	–	30	16/32	43,095	NA	NA
M40	2	+	+	35	13/15	61,976	hypothetical protein	glucose-6-phosphate isomerase
M81	62	+	+	25	8/9	36,936	hypothetical protein	secretory peroxidase
M31	6	+	+	55	12/12	51,423	hypothetical protein	UDP-glucose pyrophosphorylase
M65	7	+	+	25	4/5	45,939	hypothetical protein	isocitrate dehydrogenase (NADP+)
M32	46	+	+	25	5/8	10,115	NA	NA

¹ Percentage of donors who responded to at least 1 peptide from these antigens (n = 20).

² Number of peptides that elicited an IL-5 response in at least 1 donor out of all peptides tested for that antigen.

counting for 40% of the total IL-5 response) or known (20 peptides accounting for 90% of the total IL-5 response) antigens were performed after 14-day expansion cultures. These assays revealed that T cell responses in the memory population were much greater compared to the naïve population. Finally, responses to both the novel and known peptide pool could also be detected directly ex vivo in sorted Th2 (CXCR3–CC4+) and Th1 (CXCR3+CC4–) cells.

These data confirmed our hypothesis that the reactivity gap observed between known allergens and whole Tg extract is explained by additional T cell antigens, some of which are not targeted by IgE or IgG. Thus, the universe of allergenic T cell antigens expands well beyond those proteins that are targeted by IgE responses. This combined unbiased transcriptomic, proteomic and immunomic approach (as outlined in fig. 1) is also applicable to other allergen sources, thus providing the potential to greatly broaden the repertoire of protein antigens targeted by T cells directly involved in type I allergy. Furthermore, our data demonstrate that unlinked T cell help is

operational in Tg-specific immune responses, providing a novel insight into the immunological mechanisms associated with allergic reactions.

Benefits and Limitations of Current Specific Immunotherapy Regimens

The discovery of proteins that induce strong Th2 responses but are not targeted by IgE suggests the exciting possibility of new targets for safe and effective immunotherapy, which is to date the only causative treatment of allergic disease [34, 35]. Allergen avoidance, albeit effective, is most often not feasible, especially so in the case of pollen allergies. Hence, effective treatment of allergic manifestations is required. A variety of drugs are available to manage day-to-day allergic symptoms. Pharmacobased treatments have, however, no direct effect on the immunological processes involved in sustaining IgE sensitization and allergy but rather they interfere with differ-

ent immunological processes, such as mast cell degranulation, neutralization of certain mast cell mediators, suppression of T cell activation, general anti-inflammatory activity and/or reverse vasodilation and bronchoconstriction [36].

Because of their symptomatic nature, drugs effective in treating allergic disease rely on repeated administrations, and a more permanent treatment addressing the underlying cause of the disease is desired. In this context, specific immunotherapy (SIT) provides an effective alternative. SIT typically involves either subcutaneous or sublingual administration of allergen extract, and has been shown to provide long-term benefits that typically persist even after treatment is discontinued [34, 37].

SIT has been utilized for over a century [38] and it remains to be the only allergen-specific, disease-modifying treatment available to this day [39, 40]. For grass pollen allergy, both subcutaneous and sublingual SIT have been shown to induce immunomodulatory effects that effectively relieve patients of their symptoms [41, 42]. Although the exact mechanisms of SIT remain a subject of discussion, several hallmarks of treatment have been identified, including the induction of blocking IgG4 antibodies, IL-10-producing T regulatory cells and a reduction in Th2 responses [43–46].

Although SIT is an established method to effectively treat allergic disease in many patients, it is also associated with certain limitations mostly due to incomplete efficacy and safety concerns. Injections or oral uptake of the whole, unfractionated allergen extracts currently used for SIT does bear the risk of induction of adverse IgE-mediated reactions. As a result, SIT for patients at high risk (i.e. asthmatic patients with $FEV_1 < 70\%$) is not recommended, even though they are arguably the ones in highest need of an effective treatment. Accordingly, new approaches for the development of a safer but equally or more effective immunotherapy are required.

Strategies for the Development of Safer SIT

Adverse side effects associated with SIT most commonly result from IgE-mediated immunological events. It has been demonstrated that successful SIT is accompanied with measurable modulation of allergen-specific T cell responses [21, 43, 45]. Therefore, allergenic proteins have been engineered to include chemical alterations in the critical residues for IgE binding, while at the same time retaining their T cell reactivity. These recombinant allergens are commonly referred to as hypoallergens. Hy-

poallergenic variants have been made for several Tg allergens, including Phl p 1 [47] and Phl p 5 [24, 48, 49].

Another approach to decreasing the risk of adverse reactions during SIT administration is the production of 'allergoids', a term to describe allergens that have been modified using chemicals, such as formaldehyde or glutaraldehyde, for treatment [50–52]. Hypoallergenic allergoids do present benefits and can achieve effective immunomodulation. However, the standardization and characterization of allergoid extracts have proven problematic because the potency of allergoids and the amount of major allergens in these complexes cannot be measured after the chemical modification.

An alternative approach, which is focused on improving the safety of SIT by removing IgE binding sites in order to reduce IgE-mediated immediate type reactions, is the use of T cell epitope-based peptides [53–55]. Peptides 12–15 amino acids in size are in general too short to bind or cross-link inflammatory cell-bound IgE but still retain their ability to target T cells. Initial studies performed in cat-allergic patients showed modest clinical efficacy but were also associated with frequent adverse reactions [56]. More recent trials employing larger mixtures of shorter peptides achieved more significant reductions in early- and late-phase allergic reactions following allergen challenge [57]. In those studies, immune modulation in the form of reduced Th1 and Th2 cell responses and enhanced IL-10 production was observed. Recently, Spertini et al. [58] employed an approach in which 3 selected contiguous overlapping peptides derived from Bet v 1 that lack IgE reactivity were used to treat birch pollen-allergic patients. The initial trial, which included 20 patients (15 = active injections, 5 = placebo), produced promising data with regard to the safety and efficacy of this treatment. Although peptide-based allergen-specific immunotherapy is a very promising new approach that may provide better safety for cases where adverse reactions to SIT may be a real limitation, further research is needed to demonstrate its efficacy at inducing long-term clinical tolerance for different allergies. In addition, peptide immunotherapy relies on the definition of T cell epitopes of the allergen of interest. Despite considerable efforts in the field of T cell epitope identification for different allergens, this may still be a limiting factor for certain allergen-specific peptide-based immunotherapy approaches, and further research is needed to bridge those gaps.

In addition to avoiding IgE-binding and specifically targeting T cells, the role of antigen-presenting cells and their potential use in guiding the allergen-specific response away from Th2 towards responses dominated by Th1 and T_{reg} phenotypes are also currently actively inves-

tigated. Toll-like receptor ligands are used in combination with allergens to shift the Th1/Th2 balance [59–61]. Ongoing clinical phase II and III trials generated encouraging data and suggested potential clinical efficacy of different adjuvants [reviewed in ref. 62].

In conclusion, SIT regimens are established as effective treatment for patients suffering from several IgE-mediated hypersensitivities, including hymenoptera venom allergy [63], allergic rhinitis [64] and allergic bronchial asthma [65]. However, several limitations regarding safety, applicability and standardization remain. The ultimate goal still to be achieved is to develop a treatment with well-defined molecules or complexes associated with equal or higher clinical efficacy, while not being associated with adverse side effects. Some of the novel Tg antigens identified by us have immunological characteristics (lack of IgE reactivity but targeted by T cells) that make them potential candidates for a therapeutic approach.

Novel Tg Antigens as Targets in SIT

As described above, improvement in the safety and efficacy of current SIT is highly desirable. Data generated during the discovery and characterization of the novel Tg antigens showed that a large subset of the proteins identified lacks the ability to bind IgE while retaining the capacity to activate T cells from allergic donors. Indeed, these are potentially highly desirable characteristics for potential SIT candidate proteins.

To assess the potential of the novel Tg antigens as candidates for SIT applications, we sought to characterize the T cell response of donors who had received SIT against the novel Tg peptides and subsequently compare them to responses from the independent allergic cohort [66]. Mirroring our previous study, peptide pools were screened for T cell reactivity by ELISPOT using Tg extract stimulated in 14-day *in vitro* cultures. The most striking observation revealed by this screen was a pronounced decrease in IL-5 production for both known Phl p allergens and novel Tg antigens [66]. No difference in IL-10 production was detected, which was somewhat unexpected in light of previous observations [43, 67]. However, this may be related to the fact that IL-10 production is often associated with early stages of SIT [43] and is not always found to persist in the maintenance phase associated with SIT [68].

The reduction in IL-5 production observed for the novel antigens was more pronounced in some antigens than others [66]. Accordingly, we selected a subset of 20 peptides derived from 6 antigens to constitute a pool as-

sociated with the most pronounced downmodulation of IL-5 responses in SIT-treated donors compared to allergic donors. This peptide pool was tested in a new cohort of 20 allergic and 20 SIT donors. The drastic decrease in IL-5 production previously observed in SIT donors was replicated, and concomitantly a highly significant increase in IFN γ was detected. Again, no difference in IL-10 production between the two cohorts was observed [66].

Encouraged by these data, we assessed if the reduction in IL-5 could be correlated with treatment efficacy. During recruitment of SIT donors for these studies, patients provided a subjective evaluation stating whether or not they felt the treatment had helped to alleviate their allergic symptoms. SIT donors were segregated into two groups depending on whether they judged SIT a success or not. When the data pertaining to the IL-5 response modulation were subsequently analyzed, as a function of these accounts, it revealed that the decrease in IL-5 was in fact much more pronounced in donors who self-reported benefit from the treatments versus those that did not [66].

Overall, these data suggest that T cell responses to selected epitopes may serve as a biomarker to assess SIT efficacy. Subsequent studies will assess T cell reactivity in a longitudinal rather than cross-sectional cohort, and include detailed study of antibody reactivity on a donor-to-donor basis and objective, clinical readouts to measure allergic symptom improvement following SIT. This will further evaluate if modulation of Th2 responses to selected epitopes follows or precedes clinical improvement, and for how long the T cell modulation is stable.

In summary, numerous novel strategies and approaches are being pursued to improve the safety and efficacy of SIT based on the complex pathogenesis of allergic diseases and the strong need for effective cures. In the work reviewed here, we successfully employed a bioinformatic-, proteomic- and immunomic-based approach to identify and efficiently characterize a set of novel Tg antigens, which may be used as potential biomarkers or even therapeutic targets for allergen-specific IT. In our opinion, this approach can be applied to other allergies and therefore lead to the discovery of a much broader spectrum of potential therapeutic targets.

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