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Biomarkers for Antigen Immunotherapy in Allergy and Type 1 Diabetes

Jared M. Odegard, **Gerald T. Nepom**, and **Erik Wambre**

Benaroya Research Institute at Virginia Mason, 1201 Ninth Avenue, Seattle, WA 98101 USA

Jared M. Odegard: jodegard@benaroyaresearch.org; Gerald T. Nepom: nepom@benaroyaresearch.org; Erik Wambre: ewambre@benaroyaresearch.org

Abstract

Allergy and type 1 diabetes are immune mediated diseases that, despite being etiologically distinct, have inappropriate activation and effector function of antigen-specific T cells in the pathogenic process. Understanding changes in frequency and phenotype of these cells is critical to improve assessment of disease diagnosis and prognosis and effectively assess immunological response to therapy. In the setting of antigen-specific therapy in allergy and type 1 diabetes, assays to monitor the immunological mechanisms of disease have been improving in recent years, and we are getting closer to an accurate understanding of how the cellular immune response is modulated during treatment. In this review, we summarize the current state of cell-based immune monitoring of antigen therapy trials. We then discuss emerging advances in antigen-specific biomarkers that are transforming our knowledge about allergy and that have the potential to dramatically impact our understanding of T cell-mediated autoimmune diseases, such as type 1 diabetes.

Keywords

Allergy; type 1 diabetes; biomarkers; pathogenesis; immunotherapy

1. Introduction

Allergy and type 1 diabetes (T1D) are complex immunological disorders with multiple cellular and molecular alterations in pathways involving both activation and effector function. To rationally evaluate the mechanistic impact of candidate therapies in these diseases, therefore, it will be essential to illuminate stages of pathogenesis with the help of informative biomarkers. Biomarkers have potential applicability in multiple phases of drug development and clinical practice (Table 1). Specific opportunities to develop correlates of immune mediated disease outcome include inappropriate expansion of cells specific for intrinsic or extrinsic antigens, increased or unregulated effector functions of pro-inflammatory cells as a whole, and alterations in gene expression pathways reflecting

Correspondence to: Gerald T. Nepom, nepom@benaroyaresearch.org.

defective homeostasis. Biomarkers directly related to pathogenesis are currently employed in both allergy (IgE and basophil activation) and T1D (hyperglycemia measures and c-peptide levels), but in both cases there remains a need to accurately measure the cellular immunopathogenic process, as well as pharmacological responses in the context of a therapeutic intervention. In other words, clinical utilization of biomarkers that directly assess pathogenic mechanisms is fundamental both for improving clinical trials and for generating new concepts for intervention. Biomarker assays used to assess clinical trial outcomes must have appropriate performance characteristics (Table 2), so it is important to consider early on whether a candidate assay will be amenable to validation.

Both allergy and T1D offer the opportunity to focus on a key step in disease pathogenesis — namely, the antigen-specific T cell response. In both cases, therapy with specific antigen is designed to perturb the pathogenic response and restore homeostatic balance, but in neither case is there yet a qualified immunological measure that predicts clinical outcome. Clinical trials designed to achieve antigen-specific tolerance, whether desensitization protocols with allergens or tolerogenic immunization with T1D autoantigens, create opportunities to develop such biomarkers by specifically measuring cellular immune responses to individual disease-associated antigens. Technical advances over the last decade with major histocompatibility complex (MHC) tetramers, multiparameter flow cytometry, and gene expression profiling have dramatically enhanced the quality and quantity of information that can be obtained regarding specific T cell immunobiology. Although still technically challenging, it is now possible to interrogate antigen-specific responses in the peripheral blood of patients in antigen therapy trials, revealing new insights into disease pathogenesis and creating new biomarkers for evaluation.

2. Antigen-specific biomarkers in allergy

Figure 1 presents a schematic view of allergic responses, highlighting measurable checkpoints for disease progression. The core features of this model apply to many immune mediated and autoimmune diseases, in which individuals at increased risk undergo a step-wise sequence of cellular activation and maturation events that lead to effector responses directly implicated in pathogenesis. Existing clinical markers of allergic inflammation focus on IgE antibodies and histamine release by mast cells and basophils [1,2], which are the downstream effectors of allergic symptoms. The use of circulating allergen-specific immunoglobulin and basophils as surrogate biomarkers for clinical efficacy has been intensively investigated [3–5]. Specific IgE is not generally considered appropriate for monitoring immunotherapy in allergy, as the decrease in serum IgE level is modest and occurs late in treatment. It has been suggested that the presence of specific IgG4 may be associated with successful therapy [5], but this correlation is not always present, suggesting that IgG4 levels may merely reflect high allergen exposure rather than a tolerant status [6]. As clinical symptoms are mediated by allergen and IgE-dependent histamine release by basophils, assays assessing antigen-dependent activation of basophils, such as through measurement of CD203c [7] or diamine oxidase [8], have also been suggested to correlate with treatment efficacy. In allergic rhinitis, blood eosinophil counts [9,10] and serum levels of tryptase, eosinophil cationic protein [11], and osteopontin [12] have also been proposed as surrogate endpoints for therapy. While each of these candidate biomarkers merits further

investigation, they all relate to the end stage of the allergic chain reaction and therefore lack sufficient sensitivity to predict the onset of atopic disease and to predict early efficacy in therapeutic trials.

New technologies are emerging, which are capable of comprehensive analysis of genes, transcripts, proteins, immune cells, and other significant biological molecules designed to discover biomarkers upstream in the allergic disease process (Figure 1). For instance, molecular changes at the level of dendritic cells (DC) have been recently described for response-monitoring at the early stages of allergen immunotherapy with the use of label-free mass spectrometry approaches [13]. In this elegant study, Zimmer and colleagues demonstrate that expression of complement component 1 and Stabilin-1 may represent an early signature predictive of clinical tolerance during therapy and suggest that these proteins themselves may play a role in the desensitization process. Similarly, technical advances in polychromatic flow cytometry have now enabled a more detailed phenotypic evaluation of a multitude of immune cell subsets that may causally correlate with treatment efficacy. Innate lymphoid type 2 cells (ILC2), for example, have been recently reported to decrease dramatically after successful grass pollen subcutaneous immunotherapy in patients with seasonal allergic rhinitis [14].

An additional approach focused on pivotal early stages of disease is to use the frequency and phenotype of allergen-specific CD4 cells as a clinically meaningful biomarker in allergy. T lymphocytes drive allergic sensitization, primarily through a Th2-biased response pathway initiated by soluble mediators, such as thymic stromal lymphopoietin (TSLP) and IL-33. As shown in Figure 1, there is a pivotal role for T cell-derived cytokines, such as IL-4 and IL-13, in driving downstream effector responses, including eosinophil activation and B cell production of allergen-specific Ig. Since T cell activation and commitment to the Th2 lineage precede the main effector phases of allergy, biomarkers that detect allergen-specific early Th2 induction have the potential to offer both early, potentially pre-symptomatic, diagnosis and improved assessment of prognosis, particularly in the context of specific immunotherapy. We have focused on this objective, based on the notion that such cells are highly specific for the antigen and are therefore more likely to directly reflect the causative events of disease compared with biomarkers of more downstream events. A major impediment to the use of allergen-specific T cells as a clinically useful biomarker is their low frequency in peripheral blood and the lack of a validated method for their identification and discrimination from overall non-pathogenic Th2 cell types. However, the recent advances in peptide-MHC class II (pMHCII) tetramer staining has now allowed reliable and direct ex vivo visualization of antigen-specific CD4 T cells [15,16]. This analysis can be combined with large panels of phenotypic markers that now allow more biological information to be extracted from such rare cells (Figure 2). Additionally, isolation of pMHCII-binding cells provides the capacity to search for molecular biomarkers in an unbiased manner by using single cell transcriptome analysis. Using this approach, we recently demonstrated that allergen-specific Th2 cells are confined to allergic individuals and their disappearance is indicative of clinical responses induced by allergen-specific immunotherapy[16,17]. In addition, we have shown that CD27 expression on allergen-specific T cells predicts successful clinical outcome in allergen-specific immunotherapy [18,19]. Some challenges to the broad application of pMHCII tetramer technology in clinical

development continue to be low target cell frequency, the presence of multiple allergic protein components, and the difficulty in producing class II MHC-based reagents. With these limitations in mind, we recently demonstrated an alternative approach based on characterization of an allergic disease-related phenotype shared among all allergen-specific Th2 cells (CD4+, CRTh2+, CD161+, CD49d+, CD27-, and CD45RB-) [20] (and manuscript submitted). Remarkably, the proportion of these allergy-prone Th2 cells was extremely low in non-atopic individuals compared with allergic individuals, and these cells were preferentially deleted during successful allergen-specific immunotherapy, suggesting a possible role in the pathogenesis of the disease and in disease severity. As such, we have denoted the pathogenic subpopulation of Th2 effector cells, virtually unique to atopic individuals, as the Th2A cell subset [20]. The application of this technique has the potential to transform our ability to profile allergen-specific Th2 cells with the goal of illuminating biology and utilizing clinical biomarkers in allergy (Figure 3). Therapeutic strategies directed against molecular targets found in the Th2A subset will shift the focus of treatment to pathogenic steps earlier in the disease process, and these studies are now supported by assays specific for the Th2A biomarker.

3. Antigen-specific cellular immunophenotyping in T1D

There are several parallel elements and corresponding lessons to be learned when comparing immune monitoring approaches in allergy and T1D. In T1D current surrogate markers for disease progression include metabolic parameters associated with hyperglycemia, insulin-secretory capacity of islet cells as measured by circulating c-peptide, and the presence of autoantibodies specific for beta cell antigens. As in allergy, these assessments predominantly measure late stage outcomes and are poor representations of the immunological status that directly drives the autoimmune response against islet cells. This issue is particularly vexing because the goal of therapeutic intervention in autoimmune disease, such as in T1D, is to restore tolerance to autoantigens while sparing the function of T cells specific for pathogens or cancer antigens. Unlike standard immunosuppressive interventions, this goal is likely feasible only by targeting specific cells directly through antigen immunotherapy. There are at least five beta cell antigens that are commonly targeted in T1D patients: insulin, GAD65, IA2, IGRP, and ZnT8. Since 1994 to the present, various forms of two of these antigens, insulin and GAD65, have been used in clinical trials, as summarized in Figure 4. These trials have been evaluated in the treatment mode, with the goal of maintaining residual endogenous beta cell function, or in the secondary prevention mode, with the goal of preventing the progression of disease in individuals with early autoimmunity prior to complete loss of beta cells. While there have been interesting post hoc findings in subgroups of these trials, none has consistently met its primary efficacy endpoints. Of even more concern, few of these trials utilized immunological biomarkers that effectively characterized the T cell response, so our ability to understand the failure to achieve therapeutic benefit is lacking. Many variables in the therapeutic approach remain untested, and the mechanistic rationale for antigen administration in any particular formulation or regimen is weak. Thus, it is evident that we cannot advance rationally toward an effective autoantigen therapy via large efficacy trials and clinical outcome endpoints alone. A set of validated biomarker

assays focused on the quantity and quality of islet antigen-specific T cells is needed to assess directly whether candidate therapies are achieving their mechanistic goals.

Several assays have been employed in an attempt to measure islet-responsive T cells in the context of antigen-specific therapeutic clinical trials, as summarized in Table 3. The first, T cell proliferation based on incorporation of ^{3}H-thymidine, was one of the earliest techniques employed in cellular immunology. The findings from this assay can essentially be classified into two results: enhanced or suppressed proliferative responses as a consequence of antigen therapy. In trials of intranasal insulin [21] and subcutaneous insulin B-chain treatment [22], reduced insulin-specific proliferation was observed in the active arms, which could be evidence of deletion, margination, or alteration of the responsiveness of insulin-specific cells. Interestingly, in a study of a subset of diabetes prevention trial-type 1 participants, treatment with parenteral insulin was also associated with reduced proliferation in response to a range of antigenic islet cell extract fractions [23]. This result may suggest the occurrence of tolerogenic epitope spreading, though the specificity of these responses remains to be defined. In clear contrast to these trials, treatment with Diamyd® GAD-alum clearly enhances GAD-specific proliferative response, likely due to the presence of adjuvant [24]. Other methods for assessing T cell proliferation, particularly CFSE dilution, have also been studied in the context of T1D [25], but it remains to be determined whether CFSE-based methods will be consistently sensitive enough for islet antigen trial monitoring.

A major limitation of proliferation assays is that they do not provide information about the functional characteristics of the responding cells. Among antigen-specific assays that assess function, ELISPOT is by far the most validated and widely used in the infectious disease vaccine and cancer immunotherapy fields. This assay was also used to show evidence of induced tolerance to insulin, with a reduction in specific IFN-γ secreting cells after three months of intranasal insulin treatment [26]. An earlier trial by the same group, however, did not achieve the same effect [21]. In a small study of intradermal injection of a single proinsulin peptide, there was no change in peptide-specific IFN-γ, IL-4, IL-5, or IL-13 by ELISPOT, although there was anecdotal evidence of transient increases in IL-10^{+} cells in the lower dose arm [27]. A limitation of ELISPOT is the number of cytokines, one or two, that is measurable in a single assay, which limits the amount of information gained per sample. Other T cell cytokine assays, such as intracellular cytokine staining or cytokine capture assays, have not been reported for antigen therapy trials in T1D. Flow cytometry-based assays generally have lower sensitivity than ELISPOT, putting them at a disadvantage for detection of rare T cells with anti-islet specificities. A complementary approach that has been effective in quantifying multiple cytokines at once is multiplex cytokine analysis, such as Luminex, on stimulated cell supernatants. While this approach assesses the stimulated population as a whole, rather than quantifying the number of responding cells, it has been effective in generating qualitative information about the T cell response induced by GAD-alum therapy. In a Swedish Phase II study, a subset of subjects receiving two GAD-alum treatments showed highly significant increases in a broad range of GAD-specific cytokine responses, including IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ, and TNF-α [28]. In long term follow-up of these subjects four years later, another Luminex analysis showed similar results with the addition of elevated IL-1β and IL-2, but not IL-6 [29]. In a subsequent Phase III trial, the investigators employed a modified assay with an extended 7-day culture period.

A GAD-alum-treated group showed dose-dependent increases in the same array of GAD-induced cytokines, with the Th2-associated cytokines notably increasing in prevalence with the third and fourth doses [24]. Unfortunately, as these trials have not shown consistent evidence of efficacy, it is not clear whether an islet-specific Th2 response in this setting is clinically beneficial.

An alternative approach taken to assess the phenotype of autoantigen-specific T cells has been to measure antigen-induced changes in surface markers or gene expression of the entire lymphocyte population as a reflection of the characteristics of the responding cells. This strategy was also used in context of GAD-alum, where findings included treatment-associated gene upregulation (e.g., PD-L1, CD25, IL-2, Foxp3, IL-15R, and TGF-β) [28,29] and induction of $CD25^{+}$ $CD127^{-}$ and $FOXP3^{+}$ cells after GAD stimulation [28,30]. These data suggest that GAD-alum therapy, in addition to promoting the activation of effector T cells, particularly those with a Th2 phenotype, may also expand GAD-specific regulatory T cells. Confirming these findings with more direct methods will be crucially important.

A more direct method for characterizing T cells with a defined specificity in T1D, analogous to the methods discussed above in allergy, is using MHC-peptide multimers [31–33]. A recent innovation in this area, the Diab-Q Kit, uses a combinatorial labelling approach to allow monitoring of multiple CD8 islet-antigen specificities simultaneously [34]. This assay was used to analyze patients receiving Bayhill BHT-3021, a plasmid vector encoding proinsulin. Reductions in proinsulin-specific CD8 T cells were observed in a subset of plasmid-, but not placebo-, treated patients that were tetramer positive at baseline, and this change in specific CD8 T cell frequency appeared to correlate with maintenance of c-peptide [35]. Though they are approximately 10-fold less frequent than in the CD8 compartment, islet-specific CD4 T cells can also be directly measured by pMHC multimers. For specificities with frequencies below approximately 50 per one million CD4 T cells, the range in which islet antigen reactivity is typically found, it is critical to use magnetic enrichment to allow reliable detection above background [36]. The combination of fine specific frequency and phenotypic data provided by pMHC multimer assays make an attractive approach to monitor antigen-specific immunotherapy in T1D, using the same strategies outlined in Figure 2. One limitation to the pMHC multimer strategy is the requirement for known immunodominant peptide responses mapped for each relevant HLA type. While this limitation is not a barrier to effective use in a proof-of-mechanism setting focused on HLA-restricted populations, an HLA-independent assay would be ideal for downstream use as a surrogate endpoint. Assays based on the induction of surface activation markers, such as CD137 [37] or CD154 [38], on antigen-specific cells are also compatible with magnetic enrichment and could employ stimulation with overlapping peptide libraries or whole antigen. Outcomes from this type of assay have not been reported yet in the context of clinical antigen-specific therapy in T1D, but we expect these methods to be improved and highly informative in future trials.

4. Concluding remarks

We are now in an era with an ever expanding number of potential therapeutic approaches to specifically modulate antigen-specific T cells in allergy and autoimmune diseases, such as

T1D. More than ever, there is a critical need to develop and validate biomarker assays that will guide target selection, streamline the evaluation process, and expedite the path to regulatory approval. With antigen-specific T cells at the core of the pathologic process in these diseases, development and validation of assays that reliably assess both the quantity and quality of these cells will provide the key data to move rationally from antigen therapy concepts to clinical application.

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Abbreviations

T1D	type 1 diabetes
MHC	major histocompatibility complex
DC	dendritic cells
ILC2	innate lymphoid type 2 cells
pMHCII	peptide-MHC class II
TSLP	thymic stromal lymphopoietin

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Highlights

- Discovering and understanding biomarkers informs multiple phases of drug development and clinical practice.
- Biomarker assays used to assess clinical trial outcomes must have appropriate performance characteristics and require validation.
- Clinical trials designed to achieve antigen-specific tolerance create opportunities to develop biomarkers by specifically measuring cellular immune responses to individual disease-associated antigens.

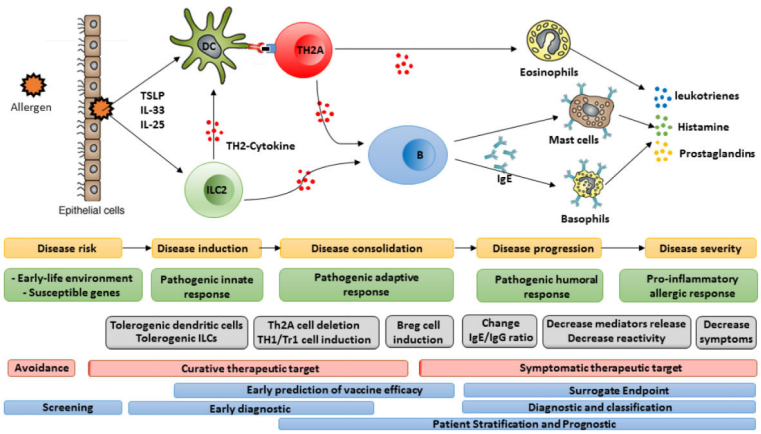

Figure 1. The process of type 1 allergic disease pathogenesis, showing opportunities for identifying biomarkers

Upon allergen recognition, epithelial cells release cytokines, such as IL-25, IL-33, and TSLP. Activation of ILC2 amplify and coordinate local immune responses. Allergens are captured by antigen-presenting dendritic cells or macrophages, and allergen-derived antigens presented by these cells are recognized by CD4+ T cells, which proliferate and differentiate. Primed allergen-specific Th2 cells and release of Th2 cytokines activate eosinophils and trigger the maturation of antigen-specific B cell populations into plasma cells. Plasma cells release antigen-specific IgE, which binds to IgE receptors on mast cells and basophils, initiating downstream histamine release when cross-linked with antigen. Key: Yellow and green boxes show the process of type 1 allergic disease pathogenesis. Grey boxes show potential treatment response markers on allergic disease process. Red boxes show principal targeted cells during current potential therapy. Blue boxes show potential types of biomarkers in an allergic disease process.

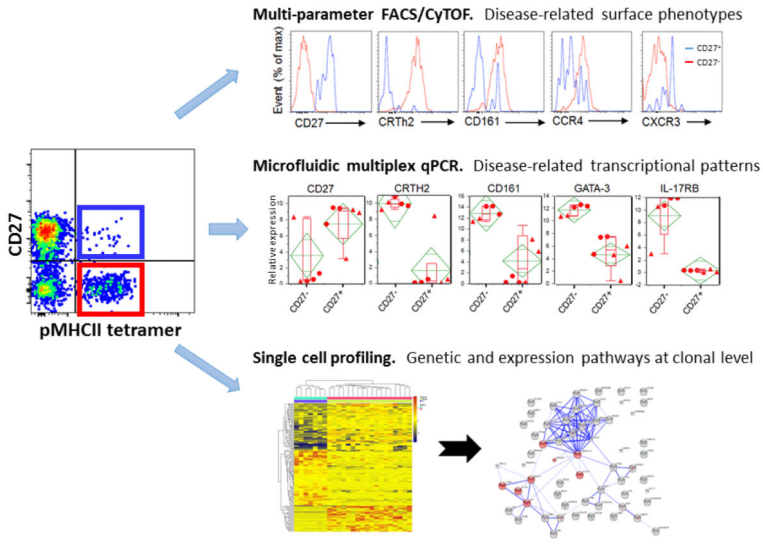

Figure 2. Strategies for discovering allergen-specific CD4+ T cell biomarkers

MHC class II tetramer technology enables technological advances in immunological research that foster biomarker discovery. Multi-parameter flow cytometry and mass cytometry provide us the ability to search for cell-surface marker signatures within pMHCII tetramer-positive CD4+ T cells. Simultaneously, we can use isolated antigen-specific CD4+ T cells to search for molecular biomarkers by micro-scaled RNA sequencing or single cell RNA sequencing.

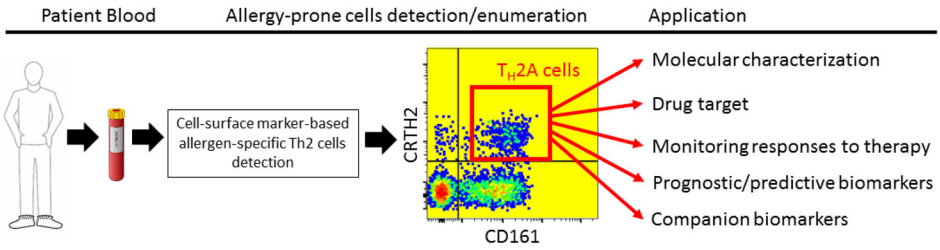

Figure 3. Illustration of potential applications of cell surface marker-based allergen-specific Th2 cells

Blood samples are collected from patients and processed to allow detection of whole allergen-specific Th2 cells (Th2A subset), based on an allergic disease-related phenotype shared among these cells. Enumeration of Th2A cells can then be applied as a clinically useful biomarker in allergy, while these cells can be purified for more molecular profiling and analysis in cell culture.

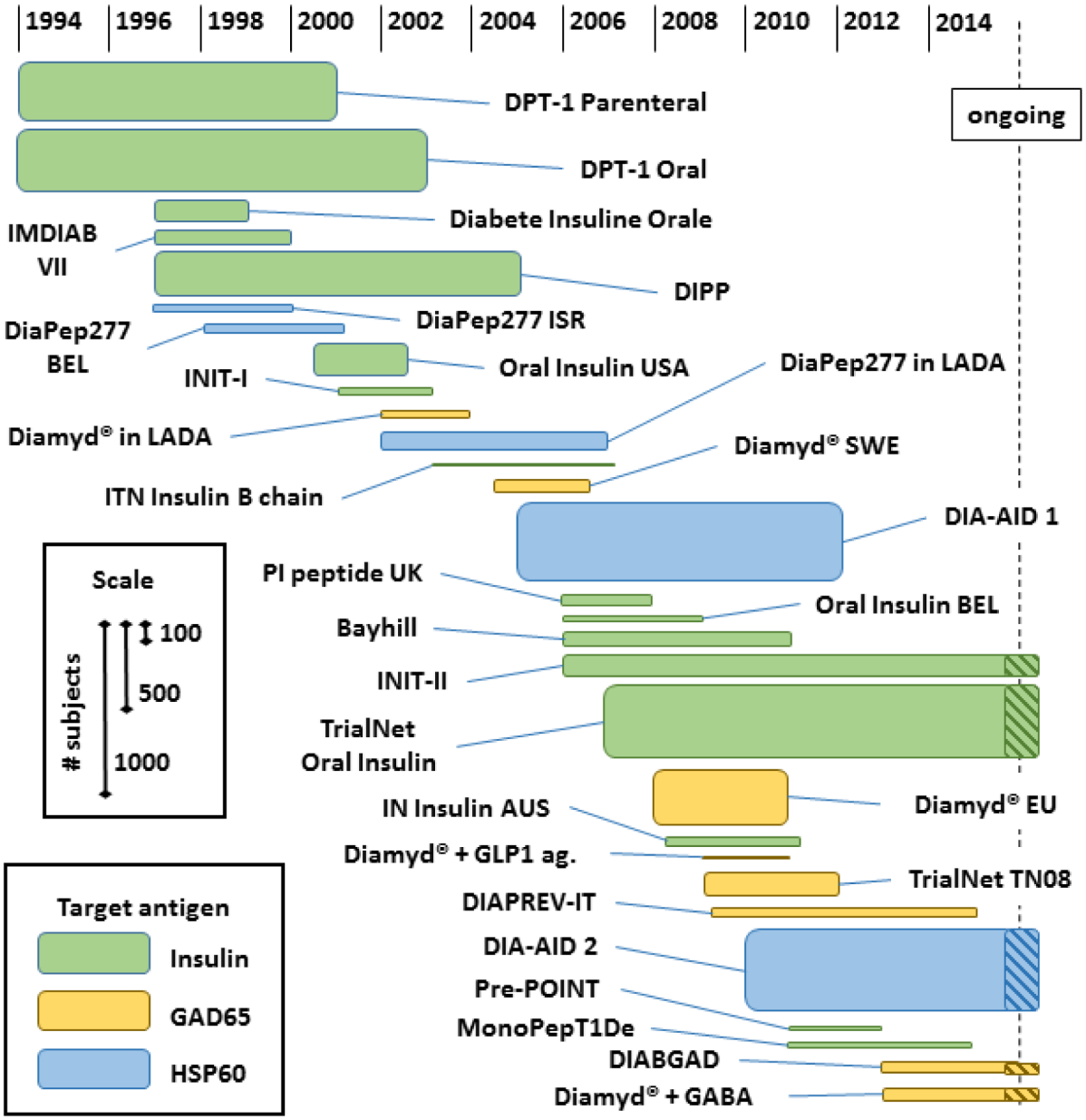

Figure 4. Antigen immunotherapy trials in T1D

Trial data, including enrollment number, start date, and primary endpoint stop date, are determined from literature references, the website www.ClinicalTrials.gov, and EudraCT. Estimates are made when specific data were unavailable.

Table 1

Types of biomarkers

Biomarker Class	Clinical Use
Diagnostic	Indicate presence of a disease
Prognostic	Indicate likely disease course if untreated
Companion Diagnostic	Identify patients likely to respond to a specific therapy
Pharmacodynamic	Identify pharmacological response to treatment
Screening	Identify patients at risk for disease development
Surrogate	Substitute for clinical outcome in efficacy trials

Table 2

Performance characteristics for analytical methods

Characteristic	Definition
Feasibility	Utilizes accessible biological tissue and possible to scale up for routine use
Precision	Closeness of test values to one another when analyzing the same specimen
Accuracy	Closeness of test values to the true value
Specificity	Ability to measure only the intended analyte, particularly in the presence of other entities
Sensitivity	Lower limit of quantitation within a defined degree of confidence
Robustness	Assay performance maintained across sites, operators, instruments, and reagents

Table 3

Cellular biomarker assays in antigen immunotherapy for T1D

Assay	Trial	Agent	Treatment effect
3H-thymidine incorporation	INIT-I	Insulin	↓ insulin response vs. placebo [21]
	DiaPep277 BEL	HsP60 peptide	Negative change in HSP60 response associated with better outcome [39]
	ITN Insulin B chain	Insulin B chain	↑ insulin B chain response vs. placebo [22]
	DPT-1 Parenteral	Insulin	↓ response to islet extracts vs. placebo [23]
	Diamyd® EU	GAD-alum	↑ GAD65-specific response vs. placebo [24]
Stimulation phenotyping	Diamyd® SWE	GAD-alum	↑ GAD65-induced CD25 and FOXP3 protein, multiple cytokine transcripts including TGFβ [28]
	Diamyd® SWE	GAD-alum	↑ GAD65-induced blasting cells with activated/T_{EM} phenotype [29]
	Diamyd® SWE	GAD-alum	↑ GAD65-induced CD25+ CD127-cells [30]
ELISPOT	DiaPep277 ISR	HSP60 peptide	↑ HSP60-specific Th2/Th1 ratio in active vs placebo [40]
	PI peptide UK	Proinsulin peptide	Transient ↑ PI-specific IL-10+ cells [27]
	IN Insulin AUS	Insulin	↓ PI-specific IFN-γ^+ cells [26]
Tetramer	Bayhill	Proinsulin plasmid	↓ PI-specific CD8 frequency in subset with positive outcome [35]
Cytokine secretion	DiaPep277 BEL	HSP60 peptide	↑ HSP60-specific production of multiple cytokines [39]
	ITN Insulin B chain	Insulin B chain	↑ insulin-specific TGF-β production [22]
	Diamyd® SWE	GAD-alum	↑ GAD65-specific cytokines, mostly Th2 [29]
	Diamyd® SWE	GAD-alum	↑ GAD65-specific cytokines, mostly Th2 [28]
	Diamyd® EU	GAD-alum	↑ GAD65-specific cytokines, ↑ Th2 skewing with additional doses [24]