Immune biomarkers in immunotherapeutic trials for type 1 diabetes: Cui prodest?

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Abstract

Decades of research efforts aimed at upgrading type 1 diabetes (T1DM) treatment did not harvest much success besides improving insulin therapy, which remains the standard of care since 1922. Immunological strategies targeting autoimmune mechanisms, rather than their metabolic consequences, are highly demanded. A deal of preclinical studies in animal models offered some promises, which were however not maintained once translated into human. All these immune intervention trials evaluated metabolic and clinical endpoints, namely C-peptide secretion, HbA1c and insulin requirements. While critical, we argue that these endpoints are insufficient and should be complemented with immune surrogate endpoints, i.e. biomarkers reflecting the immune modifications induced by such treatments. This is even more critical when clinical expectations are not met, in order to sort out the reasons of such failure, i.e. whether immune changes are not accomplished or whether, despite being accomplished, they are insufficient to translate into clinical benefits. Furthermore, these ancillary analyses may give precious indications to design further trials, i.e. to enroll patients with the best odds to respond to therapy and to follow-up their response.

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1. Introduction

Type 1 diabetes (T1DM), one of the most common autoimmune diseases, results from immune-mediated destruction of pancreatic β-cells. Both humoral (i.e. antibody) and cellular (i.e. T lymphocyte) immune responses are activated in T1DM.
Islets autoantibodies (aAbs) have been associated with T1DM for 40 years [1]. However, contrary to other autoimmune diseases such as myasthenia gravis and Graves’ thyroiditis, they do not impinge on islet destruction, but they are excellent markers of β-cell autoimmunity. On the other hand, T lymphocytes (both CD4+ and CD8+) play the major role in β-cell destruction.

There is currently no cure for T1DM and the only effective treatment is still limited to lifelong insulin replacement. Curative approaches should instead aim at halting autoimmune β-cell destruction by immune interventions. Several clinical trials aimed at either preventing or treating T1DM have however been uniformly unsuccessful. These trials were based on preclinical studies, mainly performed in the non-obese diabetic (NOD) mouse model. This model develops autoimmune diabetes spontaneously without any experimental manipulation, thus offering a valuable setting which is seldom available for autoimmune diseases. We will argue about the importance of immune surrogate biomarkers to follow-up immune modifications induced by treatment.

2. What can we expect from type 1 diabetes immune intervention trials?

The first issue considered when designing T1DM immune intervention trials is that the risk-benefit balance must be carefully weighed. Contrary to immunotherapies attempted in deadly diseases such as stage IV cancers, the prognosis of T1DM is benign for several decades and life expectancy is similar to that of healthy individuals. For these reasons, antigen (Ag) vaccination strategies, although unsuccessful to date, are particularly attractive, as they present an excellent safety profile [2].

A second key point is to correctly translate preclinical findings into clinical trials. This is not always the case, as exemplified by the subcutaneous insulin treatment tested in one arm of the Diabetes Prevention Trial-1 (DPT-1) [3]. First- and second-degree relatives were enrolled based on positivity for islet aAbs and altered insulin secretion, corresponding to a projected 5-year risk of more than 50% to develop T1DM. Participants were treated with twice daily subcutaneous administration of NPH insulin plus an annual intravenous infusion for four days, for a median follow-up of 3.7 years. No protection on subsequent T1DM development was observed. When comparing this insulin regimen with the founding preclinical studies performed on NOD mice, this result is not surprising, as mice were continuously treated with insulin doses ∼200-fold higher [4]. Indeed, when we translated the human protocol back into the NOD mouse (i.e. short-term treatment at the lower dose used in clinical trials), only a delayed onset but no significant T1DM protection was achieved, even at high insulin doses [5]. Mechanistic studies suggest that the effect was mainly one of transient β-cell “rest”, with no significant immune modulation of islet-specific CD8+ T-cell responses.

The third issue is timing. In preclinical mouse models, treatment is usually started as early as possible, frequently at a time when the autoimmune progression has not yet kicked in or is only minimally advanced. However, this is not possible for human studies. Indeed, the earlier T1DM risk biomarkers available are aAbs, which witness that the autoimmune process is already ongoing. Moreover, aAbs, unless present against three or more islet Ags, do not mark a T1DM risk considered high enough to justify most preventative strategies. Indeed, in the presence of one or two aAbs, which is the most common case, the 15-year projected T1DM risk is of ∼30% and ∼70%, respectively. As a consequence, most immune intervention trials are performed at a later stage, typically shortly after T1DM onset. At this time, not only β-cell autoimmunity is floridly ongoing, but also a consistent fraction of the β-cell mass has already been destroyed. The exact fraction of remaining β-cells is a matter of debate. Studies in the NOD mouse suggest that the great majority of the β-cell mass (∼75%) is already destroyed by the time of diabetes onset [6]. A recent meta-analysis and mathematical modeling of three landmark histopathological studies of human T1DM pancreata [7–9] suggest that this is also the case in humans [10]. However, the extent of β-cell destruction varies with age, ranging from 85% in children to 40% in adults [10]. Moreover, although it is increasingly suggested that this β-cell mass may regenerate to some extent, definite evidence is lacking. For these reasons, it is clear that even the most effective immune therapy, capable of completely halting autoimmunity, can only rescue a limited number of β cells.

3. Immune surrogate biomarkers: cui prodest? What for?

In light of these considerations, the challenge facing immune intervention trials performed at T1DM onset is formidable. It is not therefore surprising that the classical clinical and metabolic endpoints used, i.e. HbA1c, insulin requirements and fasting and/or stimulated C-peptide levels, are seldom significantly modified. Moreover, these parameters can only be assessed at the end of the trial, thus taking several months or years.

There are therefore several reasons for which immune intervention trials should be accompanied by ancillary studies evaluating immune biomarkers. First, these biomarkers can change quite rapidly, already some weeks or months after treatment, thus providing intermediate endpoints before follow-up completion. Second, immune biomarkers give valuable information about whether immune modifications suggestive of immune tolerance restoration are induced by treatment. This information is even more important when no significant clinical effect is achieved, as it allows to sort out between two explanations [2]: is this due to lack of immune efficacy (i.e. no immune tolerance restoration)? Or rather, is the tolerogenic effect achieved but insufficient to translate into significant β-cell rescue? Third, ancillary studies may allow pinpointing immune biomarker profiles associated with more favorable clinical outcomes. These profiles may allow to exclude those patients who are unlikely to respond to treatment. This selection may be performed either directly at the time of enrollment, in the case of biomarkers already present before treatment, or during the trial, in the case of biomarkers appearing after intervention. Two main types of immune biomarkers can be used to this end: aAbs and T-cells.
4. Autoantibodies as immune biomarkers in immune intervention trials

aAbs remain the mainstay for classifying diabetes cases as autoimmune-mediated (type 1) and for stratifying risk in first-degree relatives. Indeed, the appearance of anti-islet aAbs is the first detectable sign of β-cell autoimmunity. The traditional aAbs targeting glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2) and insulin (known as IAA [insulin aAbs] and detectable mainly in children before the start of insulin therapy) have been more recently complemented by aAbs targeting the β-cell granule protein zinc transporter 8 (ZnT8). ZnT8 aAbs are found in 26% of T1DM subjects classified as aAb-negative on the basis of the other three traditional aAb markers [11]. The presence of multiple aAbs also increases the risk of progression to T1DM both in family studies and in population-based surveys. In family studies, positivity for three to four aAbs is associated with a risk of developing T1DM in the range of 60–100% over the next 5–10 years. Studies on the general population indicate that the predictive value of multiple aAb positivity is approaching that observed among first-degree relatives [12,13]. Therefore, the question is whether changes in aAb titers can also be used to monitor immune modifications following immune interventions.

The first issue to consider is that changes in aAb titers occur quite slowly, as the plasma cells secreting them and the memory B-cells from which they derive are long-lived. In the context of immune monitoring of clinical trials, the usefulness of aAb markers could apply either pre-treatment, i.e. to select patients based on their aAb profiles, or post-treatment, to follow therapy-induced modifications.

aAbs have been used as selection criteria in several immune intervention trials. This information is most commonly used to ensure that the patients enrolled have laboratory evidence of β-cell autoimmunity, i.e. that they are truly type 1 diabetic. However, the presence of particular aAb specificities has also been used in vaccination trials, such as those performed with insulin and GAD. The rationale is to treat with a given Ag only those patients who show aAb evidence that the administered Ag is targeted by the ongoing autoimmune response, and thus that there is a reasonable chance to therapeutically deviate this response towards a more favorable outcome (i.e. immune tolerance). For example, only patients positive for anti-GAD aAbs were enrolled in clinical trials employing subcutaneous vaccination with GAD-alum [14–16]. Indeed, there is little rationale to intervene on GAD-specific immune responses without evidence that these responses are active in a given patient. It should be noted however that formal proof for this rationale is lacking, as this would require to treat for example with GAD-alum both anti-GAD aAb-positive and -negative patients, and to demonstrate that aAb-positive patients achieve better outcomes. As the clinical efficacy of vaccination trials has been quite disappointing even in aAb-positive patients, it is unlikely that such trials will be performed in the near future. However, results from the oral arm of the DPT-I trial suggest that this aAb-based selection strategy is of relevance. In this trial, first- and second-degree relatives with a projected 25–50% risk of developing T1DM at 5 years were treated with oral insulin or placebo. Overall, there was no significant protection [17]. However, a subsequent analysis of patients with higher IAA titers at enrollment showed some protection (hazard ratio vs. placebo-treated subjects 0.57, 95% confidence interval, 0.36 to 0.89) [17], although the rate of T1DM development increased and became similar to the placebo group once treatment stopped [18]. It is therefore possible that the intervention may be more effective in these individuals due to a more active autoimmunity against insulin. An alternative interpretation could be that IAAs facilitate the presentation of administered insulin to T-cells by forming immune complexes [19].

The second potential application of aAb markers in clinical trials is to monitor their modification following immune intervention. This was performed for example in the anti-CD3 phase II trial performed by Keymeulen et al. in T1DM patients at time of disease diagnosis [20]. Despite evidence for a better preservation of residual insulin secretion in anti-CD3- vs. placebo-treated patients, there was no significant change in aAb titers. It should be noted however that the clinical efficacy of anti-CD3 treatment has not been confirmed in subsequent phase III trials [21]. Lack of modifications in aAb titers could therefore be associated to lack of clinical efficacy; or simply reflect the fact that T-cells but not B-cells are targeted by anti-CD3 treatment. Results from the recent Type I Diabetes TrialNet anti-CD20 trial favor the latter interpretation. This phase II trial evaluated rituximab, a B cell–depleting monoclonal Ab (mAb), in new-onset T1DM patients, showing promising effects on both preservation of insulin secretion and metabolic control (lower HbA1c and insulin requirements) during a 1-year follow-up [22]. In a parallel ancillary study, the effect of rituximab on multiple islet aAbs (IAA, GAD, IA-2 and ZnT8) was analyzed [23]. A significant effect on IAA titers was observed: 40% of rituximab-treated IAA+ patients became IAA-negative vs. 0% of placebo-treated ones. Importantly, this effect was achieved despite concomitant insulin treatment, which commonly boosts Ab responses against exogenous insulin.

Ag vaccination trials also invite to analyze changes in humoral immunity not in terms of aAb responses, but rather of Abs, which develop against an exogenous Ag following its therapeutic administration. This has been well documented in all the GAD-alum vaccination trials performed. Subcutaneous administration was followed by a steep rise in anti-GAD Ab titers. Titers peaked at 3 months and declined afterwards, but remained significantly higher than in the placebo group at 30 months [14]. A 4-year follow-up study confirmed persistence of higher GAD Ab levels in the GAD vs. placebo group [24]. This rise in Abs was GAD-specific, as it was not observed for IA-2 aAbs. More importantly, these GAD-alum-induced Abs did not inhibit the GAD65 enzymatic activity [24]. This inhibition is instead a hallmark of patients affected with the stiff person syndrome [25], in which anti-GAD aAbs affect neurotransmission by blocking glutamic acid degradation. Thus, the rise in anti-GAD Abs following GAD-alum treatment should not expose to an increased risk to develop this syndrome. Nonetheless, whether this rise should be considered as a biomarker suggestive of a favorable immune outcome is a matter of debate. On one side, Ab responses are frequently associated with a T helper
5 (Th2) deviation of T-cell responses, which represent a potentially desirable effect shifting away from more pathogenic Th1 responses. Moreover, an inverse correlation between aAb and autoreactive T-cell responses has been suggested [26]. Although this observation remains a matter of debate, the same inverse correlation has also been demonstrated in the case of nasal insulin administration to at-risk individuals. This treatment was associated with a rise in Abs against exogenous insulin and with a concomitant decline in insulin-stimulated proliferative T-cell responses [27]. On the other hand, it is known that Ag-specific Abs can also favor activation of cognate T cells, at least in vitro [19]. Thus, it seems important to interpret these changes in Ab titers to exogenously administered Ags in light of the associated changes in the corresponding T-cell responses.

5. T-cell responses as immune biomarkers in immune intervention trials

Using T-cell responses to monitor immune changes during intervention trials presents a number of potential advantages. First, T cells are the key pathogenic effectors of T1DM. Therefore, interpreting changes in their frequency or phenotype is more straightforward than for aAbs or Abs against exogenously administered Ags, which are not pathogenic per se and whose role as potential facilitators of T-cell responses or as biomarkers of Th2 deviation remains controversial. Second, T-cell responses undergo faster modifications, as different types of effector and memory T cells are probably shorter lived and change their homing behavior to target tissues, draining lymph nodes and circulation more rapidly, thus influencing their detection in peripheral blood. We have provided such evidence in new-onset T1DM patients by following both aAb and T-cell responses both at diagnosis and after a median follow-up of 11 months. While GAD and IA-2 aAb titers were unchanged in 75% of cases, the fraction of patients displaying interferon (IFN-)γ-secreting T-cell responses specific for proinsulin and/or GAD epitopes decreased from 60–67% to 20% [28]. In this “spontaneous” setting, i.e. in the absence of immune intervention, this modification in T-cell responses may be due either to decreased antigenic stimulation due to a decline in the β-cell mass; or to a tolerogenic effect of insulin therapy, which may both act as an insulin Ag vaccination and “rest” β cells, thus making them less immunogenic.

In front of these advantages, there is also a major drawback, namely that the techniques to measure T-cell responses are more cumbersome. While aAbs rely on well-standardized biochemical assays utilizing serum samples that are easy to prepare and store, T-cell assays fulfilling the same requirements are just starting to be developed. These assays employ live peripheral blood mononuclear cells, which should be prepared and stored following procedures not implemented in routine clinical laboratories. Following the successful efforts of the Diabetes Antibody Standardization Program (DASP) over the last two decades, the T-Cell Workshop initiative of the Immunology of Diabetes Society is starting to provide guidelines on how to handle these biological samples [29]. The other key mission of the T-Cell Workshop is to launch multicentre initiatives to independently validate and standardize T-cell assays which have shown promise in single-center studies.

Another point to consider is that T-cell assays are better poised to detect relevant biomarkers when used to analyze responses against β-cell Ags rather than polyclonal T-cell responses as a whole. Arguably, analyzing T-cell responses independently of their Ag specificity is equivalent to analyzing titers of the whole Ab repertoire of a given individual. It is unlikely that treatments that are deemed to be not immunosuppressive or even Ag-specific will induce any significant change detectable at this level. If present, such changes would rather suggest that the tolerogenic effect is not selective enough and may expose to a higher risk of adverse events. To perform T-cell assays of this kind, it is essential to continue mapping the molecular targets (Ags and epitopes thereof) recognized by such T cells.

As for aAb assays, also T-cell assays could be applied to immune intervention trials to monitor T-cell responses either pre- or post-treatment. Application to the pre-treatment phase, i.e. to guide patient enrollment based on specific T-cell reactivity profiles, has not been attempted to date. Such application would be particularly relevant for Ag vaccination trials. The rationale is the same proposed for aAb profiles, i.e. to select patients with the best odds to respond to Ag administration because of active T-cell responses targeting the chosen Ag. We have assessed the potential utility of this approach in at-risk subjects treated with intranasal insulin to prevent T1DM development. We observed that this treatment efficiently blunted proinsulin-stimulated IFN-γ secretion by T cells in those subjects who had detectable responses at the time of enrollment. On the contrary, subjects that did not display such IFN-γ responses before treatment did not show any T-cell modification, i.e. these responses remained undetectable throughout the 12-month follow-up (Martinuzzi et al., unpublished). The message is two-fold: on one hand, intranasal insulin administration does not induce unwanted IFN-γ T-cell responses, but only blunts existing ones, a reassuring observation in terms of safety. On the other, it seems of little relevance to try deviating insulin-specific T-cell responses in those subjects who do not harbor them. Thus, this type of preventative trials would be better targeted by enrolling only T-cell-positive individuals.

Monitoring of T-cell responses has instead been applied to follow immune modifications after treatment. We have participated to two Ag vaccination trials of this kind. We recently reported a proof-of-concept study on aAb+ diabetic patients not requiring insulin at the time of diagnosis. These patients were treated with intranasal insulin in an attempt to save residual β cells. Although nasal insulin-treated patients eventually progressed towards insulin dependency at a rate similar to placebo-treated ones, we could document successful induction of insulin-specific immune tolerance both at the T-cell and Ab level [30]. Contrary to what observed in the placebo arm, patients treated with intranasal insulin displayed significant reductions in IFN-γ-secreting proinsulin-specific T-cell responses. This effect was Ag-specific, as it was not observed for responses towards the tetanus toxoid recall Ag. The initiation of insulin therapy further documented that this proinsulin-specific tolerance was
operational in vivo, as intranasal insulin-treated subjects failed to develop anti-insulin Abs [30].

Similar T-cell assays were applied to the Diamyd GAD-alum subcutaneous vaccination trial performed by J. Ludvigsson [24]. The results were different in this case, showing that GAD-alum, but not placebo (alum only) treatment, did not blunt, but instead selectively boosted GAD-specific T-cell responses. Moreover, these T-cell responses did not show a tolerogenic deviation towards secretion of Th2 or regulatory cytokines, as Th1 cytokines were equally produced. Furthermore, patients displaying a better clinical outcome (i.e. C-peptide decline \( \leq 60\% \)) were also characterized by GAD-induced T-cell responses more deviated towards favorable Th2 (IL-5, IL-13) and regulatory (IL-10) cytokine profiles. These results suggest that, in the case of GAD-alum vaccination, the immune modifications induced are not selectively driven towards the desired outcome. One critical parameter influencing this paradoxical boosting of GAD-specific T-cell responses and the lack of cytokine selectivity may be the use of alum adjuvant. Indeed, adjuvants are employed in vaccinology to improve Ag immunogenicity, an outcome which is opposite to the immune tolerance state aimed for in T1D.

6. Improving our immune monitoring armamentarium: islet imaging techniques

Contrary to other autoimmune diseases, direct biotic access to damaged tissues is not a viable option in T1DM, due to the anatomical position of the pancreas, to the risk of causing organ damage and to the limited islet sampling that can thus be obtained. Islet imaging is therefore a florid field of investigation in the field, not only for basic biomedical research, where different reporter gene mouse models are available, but also for clinical research. In the latter case, suitable imaging techniques should fulfill several requirements: they should be non-invasive; ideally, they should provide suitable resolution to visualize islets (which are in the size range of 50-500 \( \mu m \)), allowing both anatomical and functional imaging without prior ex vivo cell labeling.

Magnetic resonance imaging (MRI) has thus far provided the most promising results, either in mouse models or in patients. MRI is a non-invasive imaging technique that is capable of delivering high-resolution quantitative information about local tissue accumulation of selected cell types following labeling with a contrast medium. It has the potential to provide two key informations: quantification of the islet mass and infiltration by immune cells (so called insulitis).

The group of Moore has made progressive attempts at monitoring immune infiltration in the pancreas. These investigators were the first to show that it is possible to label lymphocytes with superparamagnetic iron oxide nanoparticles. They designed iron-oxide-labeled recombinant MHC reagents loaded with a \( \beta \)-cell epitope, which are capable of binding diabetogenic CD8 + T lymphocytes recognizing this epitope [31]. Transgenic CD8 + T cells specific for this \( \beta \)-cell epitope were labeled with these reagents and injected into immunodeficient NOD.scid mice. Their migration to the pancreas could thus be followed in real time, documenting their progressive accumulation [31].

This approach still requires prior ex vivo labeling of T cells before transfer. One further step was to examine if these probes could be used to label autoreactive T cells directly in vivo following intravenous injection, as this would be easier to translate into clinical application. This approach also proved feasible, as injection of contrast-labeled epitope-MHC probes allowed tracking of endogenous autoreactive T cells in NOD mice [32]. Probe accumulation in the pancreas was Ag-specific, age-dependent, and correlated with the numbers of contrast-labeled CD8 + T cells recovered from the pancreata of injected mice.

This approach has so far been attempted only in mice. Gaglia et al. applied a different MRI strategy on human subjects by intravenously injecting magnetic ferrous nanoparticles [33]. These nanoparticles accumulate in inflamed tissues due to increased vascular permeability and subsequent uptake by infiltrating monocytes/macrophages recruited at this site. These authors were thus able to document increased signal accumulation in pancreata of T1DM patients compared to healthy individuals 48 hours after nanoparticle injection. A critical control group, namely type 2 diabetic patients, is missing in this report to ensure that the observed signal alterations are specific of the insulitic infiltrates of T1DM and do not simply reflect low-grade inflammation, which is also present in non-autoimmune type 2 diabetes.

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are further non-invasive imaging techniques that provide good resolution, high sensitivity, and accurate functional quantification in living subjects [34]. Dozens of nuclear and radioactive probes are under testing for islet imaging in both rodent models and humans, including saccharide-based probes (\( ^{18} \)F-labeled fluorodesoxyglucose and D-mannohexitolose), neural imaging probes (\( ^{11} \)C-dihydroetrebazabine, \( ^{18} \)F-fluorobenzyltrozamicol, L-dihydroxyphenylalanine [L-DOPA], \( ^{18} \)F-fallypride), and radiolabeled sulfonyleureas, glucagon-like peptide 1 and mAbs recognizing molecules expressed on the \( \beta \)-cell surface [34]. While MRI has high spatial resolution but low sensitivity, PET has poorer spatial resolution but higher sensitivity. Therefore, combined PET and MRI techniques, in combination with specific imaging probes, could be very promising to obtain more complete functional and anatomical imaging of islet \( \beta \) cells [35].

These non-invasive imaging techniques may ideally provide quantitative information about pancreatic immune infiltrates and residual \( \beta \)-cell mass. Although highly promising, further studies on humans are needed before envisaging applications for monitoring immune intervention trials.

7. Conclusion

In light of the formidable challenge of rescuing a significant \( \beta \)-cell mass in patients that are already diabetic, it is relevant to evaluate not only clinical and metabolic outcomes, but also immune surrogate endpoints. This is even more important in trials not showing clinical benefit, or not poised to detect such benefits, as is commonly the case for phase I studies. Indeed, the lack of clinical effects could be open to disparate interpretation,
including late treatment in front of full-blown disease or failure to restore immune tolerance. These possibilities can be sorted out by immune monitoring analyses performed before, during, and after treatment. Comprehensive “immune staging” of T1DM could be valuable during the different steps of diagnostic, prognostic and therapeutic work-up (Fig. 1). Biomarkers of β-cell autoimmunity such as aAbs and T-cells could help identifying at-risk subjects at an early stage (preclinical diagnosis) and to follow them up over time to decide the need for immune therapy and the best timing for treatment (prognostic stratification). Ag-specific immune therapies could be tailored to each subject by administering therapeutic formulations of those Ags targeted by aAb and/or T-cell responses. Modifications induced on such responses could be followed in real time during treatment, thus allowing to assess immune efficacy prior to and independent of clinical outcome. Knowing whether Ag-specific T-cell responses are modified and in which individuals also provides key mechanistic insights to plan further trials, i.e. to optimize enrollment criteria and to modify therapeutic strategies accordingly.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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