Functional genetics of type 1 diabetes: between genes and disease

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6 GENERAL DISCUSSION AND SUMMARY

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Type 1 diabetes is an autoimmune disease that results in the loss of insulin-producing beta cells in the pancreas. The etiology of the disease remains incompletely understood, but both environmental and genetic factors contribute to disease susceptibility. Elucidating the genetic mechanisms involved in disease development will contribute to personalized medicine and is invaluable for the development of novel therapies and a potential cure.

Transcriptional regulation of the islet-autoantigen IGRP is similar between pancreas and thymus and not implicated in the loss of tolerance for IGRP in T1D

In **chapter 2** of this thesis I describe the role of differential splicing of *G6PC2* between pancreas and thymus in the development of islet autoreactivity. *G6PC2* encodes the autoantigen islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), a known target of autoreactive CD8 T cell in T1D. Previously, Pugliese et al. suggested that absence of *G6PC2* isoforms containing exon 3 and 4 in the thymus contributes to IGRP autoreactivity.[1] The thymus plays a key role during T cell development as the anatomical site where lymphoid progenitors differentiate and are educated.[2] Through both positive and negative selection a T cell repertoire is formed that can properly interact with antigen presenting cells while avoiding immune responses to the body's own tissue. Potentially autoreactive cells are eliminated in the thymus in a process referred to as central tolerance, which involves presenting tissue-specific autoantigens to developing T cells and the removal of those cells with the potential to react to these autoantigens. That negative selection contributes to the prevention T1D is illustrated by the fact that disease promoting *INS* variant associate with a quantitative reduction of thymic insulin expression, which is thought to impair negative selection of INS reactive T cells and promote islet autoreactivity.[3,4]

In 2006 differential splicing of *G6PC2* between thymus and pancreas was hypothesized to exert an effect on islet-reactivity through incomplete negative selection of IGRP-specific T lymphocytes. We initially set out to quantify the autoreactivity against *G6PC2* isoforms containing exon 3 and 4 in T1D patients, as these isoforms were supposedly lacking from the thymus. However, using *G6PC2* isoform specific primers demonstrated that actually all *G6PC2* isoform that employ conventional splice sites are expressed in the thymus, be it at considerably lower levels than in pancreatic islets. Yet, we did identify three novel splice variants that were only detectable in islet-isolates. As thymic expression of regular *G6PC2* isoforms was already near the lower detection limit of our assays, our inability to detect these in thymic tissue might have resulted from technical limitations, rather than from actual differential expression between pancreas and thymus. Further, these novel islet-specific isoforms employ unconventional splice sites and whether they actually are valid isoforms or erroneous byproducts of normal splicing remains to be determined by proteomic studies and immunohistochemistry. Yet, if proven to be translated into protein

product these isoforms would be an interesting subject for further functional studies, given their exclusivity to islet cells.

Next, we investigated autoreactivity against IGRP isoforms by quantifying IGRP autoantibody levels and frequencies of IGRP-specific, HLA-A2 restricted CD8 T cells in peripheral blood of patients and healthy individuals. Interestingly, T cells reactive against IGRP-derived peptides could be readily detected in the peripheral blood of healthy individuals, suggesting that negative selection of IGRP reactive T lymphocytes is incomplete. Yet, no significant difference could be detected between T1D patients and healthy individuals, implying that the apparent defective negative selection of IGRP-reactive T cells is insufficient development of T1D. In our study we focused on the interaction between IGRP and HLA-A2, the class I molecule that occurs with the highest frequencies among T1D patients.[5] Ideally we would have also been able to analyze T cells recognizing IGRP peptides in the context of other T1D associated HLA molecules, especially HLA-DR and HLA-DQ molecules given their strong genetic linkage to T1D development. Unfortunately, our technical platform currently precludes us from employing any HLA molecule other than HLA-A2. While it is technically possible to produce HLA class II tetramers, *in vitro* generated HLA-class II monomers are highly unstable. Their stability can be improved by covalently linking the peptide of interest to the HLA class II monomer, yet this increases the complexity of their production process and prohibits the high throughput analysis as we have performed here.[6,7] If technological advances would allow a similar approach for class II HLA molecules as we have performed here for HLA-A2, it would be interesting to determine the immunogenicity of IGRP splice variants in the context of the remaining HLA class I and class II molecules to determine their actual impact on the autoimmune response in T1D.

The presence of IGRP-specific T cells in peripheral blood of healthy individuals suggests that incomplete negative selection alone is insufficient for autoimmune disease to develop. IGRP reactive T cells can display potent cytotoxic activity *in vitro* and *in vivo*.[8,9] Therefore, the protection from β-cells lysis in healthy individuals must be effectuated by means other than thymic selection. Potential mechanism involve peripheral suppression of autoreactivity by regulatory T cells (Tregs) or inhibitory mechanisms inherent to autoreactive cells, e.g. increased activation threshold preventing autoreactive T cells from becoming activated, or activation induced cell death (AICD) causing apoptosis upon recognition of an autoantigen.[10,11] The role of the thymus in self-tolerance induction of CD4 T cells was recently shown to be dependent on the autoantigen involved.[12] Although our study was not designed to answer this question, it appears that distinct methods of self-tolerance induction exist for CD8 T lymphocytes as well and the role of the thymus in the prevention autoreactivity against IGRP is limited.

Since its discovery as an islet-autoantigen IGRP has been regarded as one of the driving antigens for disease development and progression in mice.[13-15] However, its exact role in the pathophysiology of human T1D remained controversial as the frequency of IGRP-specific CD8 T cells in the peripheral blood of T1D patients is modest compared that

to murine models.[16,17] In our study antibody titers and CD8+ T cell frequencies against IGRP and its isoforms were low and non-discriminating between health and disease. This suggests against the role of disease-driving antigen for IGRP in human T1D. Still, previous studies from our laboratory showed that IGRP-specific CD8 cells are present in human insulitic lesions and adoptive transfer of human IGRP-specific CD8 T cells into HLA-A2 transgenic mice provokes insulitis and β-cell destruction.[8,18] Thus, while IGRP does not appear to be a driving autoantigen in human autoimmune diabetes, IGRP autoreactivity seems to contribute to the etiology of T1D and clarifying its exact role it would be worthwhile.

Concluding, the difference of G*6PC2* expression between pancreas and thymus is not so much quantitative as it is quantitative. Further, it appears the role of the thymus in tolerance induction against IGRP is limited at most and the effect of reduced thymic *G6PC2* expression on the occurrence of autoimmune disease appears negligible.

Posttranscriptional control of pro-apoptotic genes by miRNA contributes to apoptosis resistance in autoreactive T cells in T1D

In T1D autoreactive CD8+ T-cells destroy insulin-producing β-cells that display their cognate autoantigen on the cell surface. Yet, as described in chapter 2 islet-reactive T-cells can be readily detected in the peripheral blood of healthy individuals. A potential difference between health and disease may be the capacity of peripheral regulatory T cells to dampen autoimmune responses or prevent them from even happening.Reduced Treg activity has been described for several autoimmune diseases, including T1D.[19]and, increased resistance for regulation by autoreactive T effector cells has been suggested as well.[20] In chapter 3 of this thesis we compared two autoreactive T cell clones isolated from a T1D patient with a T cell clone isolated from a healthy individual, all recognizing the same peptide:HLA complex and displaying comparable *in vitro* cytotoxicity. We observed that the healthy individual's autoreactive T cells could be stimulated for a finite number of times before collapsing, a characteristic not observed in autoreactive T cells obtained from a T1D patient. Therefore, we tested the hypothesis that in T1D uncontrolled expansion of diabetogenic T cells occurs as a result of failure to activate apoptosis upon repeated antigen exposure.

Transcriptome analysis of the T cells clones revealed reduced expression of TRAIL, TRAIL-R2, FAS and FASLG, members of the extrinsic apoptosis pathway, in patient-derived compared to healthy-donor-derived T cells. This was mirrored by increased expression of microRNAs (miRNAs) predicted to regulate these particular genes, namely miR-98, miR-23b and miR-590-5p. Gene specific targeting by these microRNAs was confirmed using dual-luciferase reporter assays. Finally, transfection of these microRNAs into primary T-cells reduced FAS and TRAIL mRNA expression, underscoring the functional relevance of these microRNAs in effectuating resistance to apoptosis of autoreactive T cells. Thus, we showed that the differences in proliferative capacity between autoreactive T cells

from health and disease associates with altered in expression of pro-apoptotic genes and the post-transcriptional factors regulating them.

To definitively prove that the increased proliferative capacity of autoreactive T cells from T1D patients stems from altered expression of pro-apoptotic factors and the microRNAs governing them, we set out to reverse the observed phenotypes via molecular intervention. Primary T lymphocytes are notoriously difficult to transfect, but we show that nucleofection is a viable way of introducing miRNA into primary autoreactive T cells. Unfortunately, employing this method on T-cells derived from non-diabetic donors caused cell death in the majority of cells within 24 hours after treatment. This timeframe fell short of the minimal time needed to observe an effect of miRNA treatment, as a resting/recovery phase for the cells and the time required for the miRNA to assert its effect needs to be taken into account. Another difficulty with our initial approach was that the estimated half-life for miRNAs is approximately 5 days.[21] The proliferative capacity of a T cell clone can only be reliably tested at the end of its respective restimulation cycle, which is around 10 days for the autoreactive T cell clones described here. To deal with these limitations, we opted to use virus-specific T cells that express high levels of FAS and TRAIL and are relatively resistant to the negative effects of nucleofection and measure the effect of our treatment with quantitative PCR analysis of FAS and TRAIL mRNA. By doing so we were able to provide evidence that overexpression of miR-23b, miR-98 and miR-590-5p indeed leads to reduced expression of members of the FAS and TRAIL pathways and thereby may contribute to increased survival of autoreactive T cells.

Our observations and transcriptome comparisons in this study involved a single $IGRP_{265-273}$-reactive T cell clone derived from a healthy individual and two $IGRP_{265-273}$-reactive T cell clones from a T1D patient. Autoreactive CD8+ T cell clones are extremely difficult to produce and maintain in culture without loss of antigen specificity, especially autoreactive T cells derived from healthy individuals. The clones used in this study represent the majority of IGRP-specific CD8 T cell clones available worldwide, and although the limited number of samples poses a potential sampling bias, here we have been able to reproducibly investigate the transcriptomes of autoreactive cells that were actually primed *in vivo*, and thus likely involved in β-cell destruction, with their non-pathogenic counterparts from healthy individuals. Naturally our study would have been strengthened if more clones could have been examined, particularly from the healthy individuals. We have aimed to obtain and compare IGRP specific cells directly after isolation from the blood of T1D patients and healthy individuals using a FACS-based approach in an attempt to increase the number of T cell clones for our study. Due to the low numbers of circulating autoreactive cells of a given specificity in both T1D patients and healthy individuals, using this approach we were unable to robustly test mRNA and miRNA expression in T cells with a single specificity using this approach. Yet, by comparing pooled autoreactive cells with varying specificities from recent onset T1D patients to pooled virus-reactive cells from the same individuals we were able to show a significant difference in FAS and TRAIL expression between autoreactive T cells and virus-specific T cells within a single individual,

which lends support to the notion that autoreactive T cells have impaired expression of surface death receptors and a subsequent increased resistance to apoptosis induction (unpublished data). Further, given the promising results from these preliminary data I am convinced that this approach stands to be an excellent alternative to the laborious cell-culture and may offer new opportunities to analyse autoreactive T cells directly ex-vivo in the near future.

Thus, repression of pro-apoptotic pathways by miRNAs contributes to unrestricted expansion of diabetogenic cytotoxic T-cells, implicating miRNA-mediated gene silencing in islet autoimmunity in T1D. Further analysis of the miRNA transcriptome of autoreactive cells might provide novel therapeutic options and elucidation of the mechanisms that govern miRNA expression may result in the identification of (environmental) triggers involved in T1D development.

Rare variants alter post-transciptional regulation of T1D risk genes

In chapter 4 of this thesis we investigated the possible interaction between rare genetic variants and functionality of miRNAs, which play an important role in the post-transcriptional regulation of gene expression. Genome wide association studies (GWAs) have greatly increased our knowledge on disease-associated genomic regions. However, even when taking all verified disease-associated gene variations into account we still cannot completely explain the heritability of T1D. GWAs are designed for the detection of common single nucleotide polymorphisms (SNPs). Rare SNPs and structural variations such as insertions, deletions and repeats are not analyzed with current GWAs and it is therefore hypothesized that the 'missing heritability' of complex diseases such as T1D can be explained by these types of genetic variations.

The magnitude of the missing heritability of T1D is subject to debate, with estimates for the unexplained heritability ranging from 0% to 40%.[22-25] Regardless of the size of the unexplained heritability, consensus is that common genetic risk variants are unlikely further our understanding and that future research should focus on structural and rare variants.[26]

In our proof-of-concept study we have investigated rare polymorphisms in T1D risk genes with the propensity to influence post-transcriptional gene control by affecting miRNA function. miRNAs in general are pleiotropic, with each miRNA regulating up to several hundreds of protein-coding mRNAs. In our study we limited our investigation to genes with known association for T1D susceptibility and investigated whether they were predicted to be under regulatory control by miRNAs. Using *in silico* modeling we identified miRNAs that could potentially interact with mRNA of T1D genes. With gene-targeting models we validated these predictions and demonstrated *in vitro* that rare SNPs, miRSNPs, have the capacity to modulate post-transcriptional gene control by instilling or abrogating miRNA-mediated gene silencing, a novel mechanism through which rare polymorphisms can affect T1D associated gene pathways. Although this study is a proof-of-concept rather

than a comprehensive analysis, we already describe several functionally active miRSNPs in T1D associated gene loci. Currently little is known about the biological importance of the miRNA identified in this study, i.e. miR-302a* and miR-523, and the expression patterns of these miRNA. However, knowledge on both miRNA species and genetic polymorphisms is rapidly increasing and the association between environemtal cues and miRNA expression is becoming more clear.[27] Although further research is required to establish the actual impact of miRSNPs on development and heritability of autoimmune diseases, this study provides a potential mechanism by which environmental factors can interact with genetic susceptibility factors in the development of T1D.

The CTLA 3' UTR $(AT)_n$ microsatellite is causal for the association of the CTLA4 locus with genetic susceptibility for T1D

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a surface molecule present on activated T cells that inhibits the T cell receptor signalling upon binding to its ligands CD80 and CD86. It has been hypothesized that inherited variations in the *CTLA4* gene can increase T cell autoreactivity and thereby play a role in autoimmune diseases such as T1D. Indeed, the *CTLA4* locus was among the first genomic regions to be associated with susceptibility for T1D. Yet, the genetic variant causal for the actual association, as well as the molecular mechanisms associated with increased disease susceptibility have long remained unknown. Recent fine mapping studies have shown that the region marked by the single-nucleotide polymorphism CT60 (rs3087243) downstream of the *CTLA4* 3'UTR acts as a susceptibility factor.[28,29] This CT60 polymorphism maps to a non-coding region and the mechanisms through which it would influence CTLA4 expression remains elusive. In chapter 5 of this thesis we provide evidence that a structural genomic variant, i.e. the $(AT)_n$ microsatellite located within the 3' untranslated region (UTR) of *CTLA4,* is causal for the genetic association of *CTLA4* with T1D susceptibility. First, we investigated the association of the *CTLA4* $(AT)_n$ microsatellite with the T1D risk marker CT60 . Analysis showed that CT60 status and $(AT)_n$ length were in extremely strong linkage and that *CTLA4* alleles containing longer $(AT)_n$ elements are uniquely associated the CT60G risk haplotype. Conversely, the protective CT60A haplotype was observed more frequently in association with wild-type, short, $(AT)_n$ elements. Thus, long $(AT)_n$ elements within the 3'UTR of *CTLA4* would directly correlate with increased risk for islet autoimmunity. In autoreactive T cell lines increased microsatellite length associated with a significant decrease in CTLA4 mRNA expression and direct transfer of a long $(AT)_n$ microsatellite resulted in decreased reporter mRNA expression compared to the transfer of a short $(AT)_n$ element.

It should be noted that the number of T cell lines studied we have studied here, i.e. 10 patient T cell lines and 8 control lines, is low and represents a potential limitation to our findings. Yet, it should also be appreciated that the cell lines used here are extremely rare and generation of additional cell-lines is technically highly demanding. Furthermore, the fact that a significant difference between the lines when stratified on $(AT)_n$ genotype

was still detected, despite the heterogeneous composition of the cell lines, indicates robustness of the association between $(AT)_n$ genotype and CTLA-4 expression.

In order to assess the effect of the $(AT)_n$ length on CTLA4 expression we transferred the 3'UTR of CTLA4 variants with different $(AT)_n$ lengths into a GFP reporter constructs. Sequencing analysis excluded any variation other than the $(AT)_n$ element between the generated constructs. Upon transduction of the constructs into a immortalized lymphocyte cell line we observed a significant difference on steady state GFP mRNA levels, with the construct containing a long $(AT)_n$ element showing decreased levels of reporter mRNA. The use of a long-standing immortalized cell line instead of actual T lymphocytes introduces the possibility of culture artefacts or aberrant outcomes due to differences in intracellular mechanisms, with the addition of potential genomic instability due to the process of transduction. Yet, as our results were consistent over two separate rounds of transduction, performed with separately produced virus batches, we are confident these confounders were kept to a minimum and our results reflect the actual influence of the $(AT)_n$ repeat on gene expression.

The location of the microsatellite in the 3' UTR, known for its role in post-transcriptional gene regulation, suggests that the mechanisms underlying the observed differences in mRNA expression involve altered post-transcriptional control. In our study we did not observe any significant effect of the $(AT)_n$ repeat on mRNA decay rate. While this suggests that the $(AT)_n$ repeat does not affect mRNA stability it remains possible that transcription-dependent factors have acted as rate-limiting factors in our experiment. Proteins with a very short half-life, or a RNA-silencing moieties that are consumed as a result of their actions would be equally affected by the actinomycin D treatment used to block the transcription of our GFP reporter. The actinomycin D treatment may thereby have obscured any mRNA decline that would have normally occurred *in vivo* between short and long $(AT)_n$ containing mRNA. Alternatively, the difference in mRNA levels may be the result of different transcriptional regulation, and not altered post-transcriptional control. It is known that the location of a genomic element does not necessarily correspond with its function and that genomic interactions can occur between regions that are up to hundreds of kilobases apart.[30] Therefore, while its localization suggests that the $(AT)_n$ microsattelite affects post-transcriptional regulation, possibly via short-lived regulatory molecules, our current data do not allow us to exclude long-range gene interactions or differential binding of chromosomal moieties as factors in the observed difference in *CTLA4* mRNA expression. Still, the extremely strong association between the CT60 risk variant and long $(AT)_n$ alleles, combined with the direct effect of the $(AT)_n$ repeat on mRNA expression suggests a causal role for the $(AT)_n$ microsatellite in the genetic association of the CTLA4 region with T1D susceptibility.

FUTURE PERSPECTIVES

The last two decades have provided (bio)medical scientists with a wealth of information on the genetics of complex diseases such as T1D. Understanding exactly how gene variants influence disease susceptibility and determining the interplay between environment, genetics and immunology have proven to be the next hurdles to overcome. Achieving this goal may enable us to predict disease progression, design novel treatment methods and improve patients categorization in order to provide actual personalized medicine. The contributions of this thesis are summarized in Figure 1. In short, we reposition the proposed alternative splicing of *G6PC2* between thymus and pancreas, discuss the role of the thymus in tolerance towards the putative islet-autoantigen IGRP and argue the validity of IGRP as an critical islet-antigen in T1D. Further we show that a structural genetic variant located with the 3' UTR of *CTLA4* associates with risk for T1D development and influences expression of the immune regulator CTLA-4. In addition, we show that rare genetic variants can influence microRNA mediated post-transcriptional control, identifying a novel mechanism through which genetic predisposition might interact with environmental trigger to influence (auto)immunity and contribute to familial aggregation of T1D. Finally, we show that pivotal apoptosis pathways are affected in autoreactive T lymphocytes of T1D patients and that microRNA play a vital role in this, again indicating the importance of understanding of gene-gene and gene-environment interactions in the development of T1D. Yet, our work only covers a small part of the unknown and many aspects of the functional genetics of T1D remain to be addressed. As mentioned before,

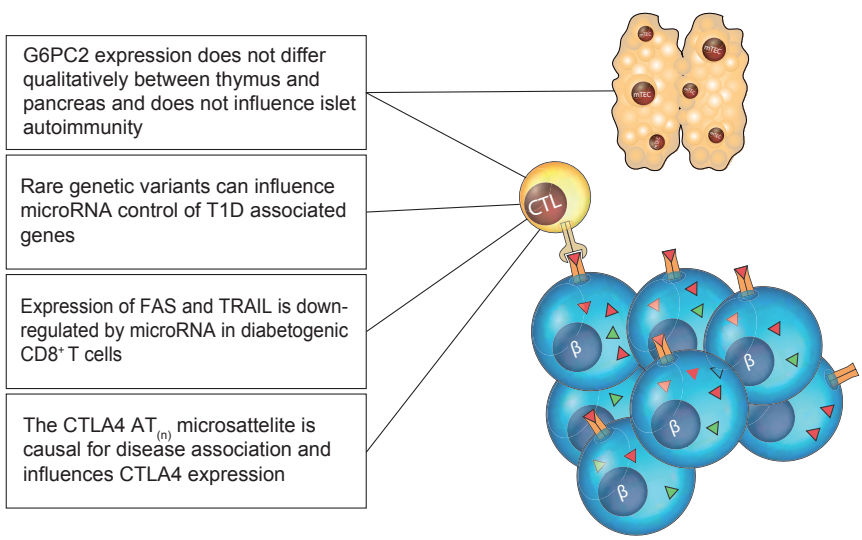

Figure 1. The immune reaction in Type 1 Diabetes – an update. Adapted from B.O. Roep, Nature, 2007[37]

despite their valuable contribution thus far, larger GWAs studies are unlikely to aid this cause further, as potential new risk SNPs are expected to carry a very modest impact on disease susceptibility at best. With current technological advances and the advent of large sequencing centers, whole-genome sequencing, a technique that charts the complete genome of a single individual, has come within reach for academic researchers. Using whole-genome sequencing, future research will be able to overcome the limitations of GWAs by assessing al genetic variation within a single individual at once. At the same time this approach will generate an unparalleled amount of complex data. Data analysis, processing and statistics will most likely prove to be a bottleneck and new strategies for data-management will be required. From the cell-biological perspective of T1D research, optimization of single cell techniques that will allow for direct isolation and investigation of autoreactive T cells directly isolated from peripheral blood can eliminate cell culture artifacts and provide 'clean' data regarding the make-up of diabetogenic T cells and what distinguishes them from their non-diabetogenic counterparts. Further, direct isolations will allow for high-throughput analysis and remove the problem of limited sample availability currently complicating research. Combined with improvements in big data analysis, this approach may prove invaluable in identifying molecular pathway involved in, or even preceding, β cell destruction.

THERAPY

The cure and complete prevention of disease occurrence is the ultimate goal in medicine, but is also be the hardest goal to achieve. Fortunately, treatment modalities are constantly improving, allowing for better patient care in the interim. The future holds several promising outlooks for management of T1D, among which the artificial pancreas[31], stem-cell derived (neo) β cells[32] and promising immunotherapies.[33-35] Recent myeloablative therapies with subsequent autologous stem cell transplantation that resulted in reduced, and in some cases very long-standing, insulin dependency demonstrate the power of immunotherapies and indicate that reprogramming the immune system may hold a potential cure for T1D [36]. Yet, the current approach subjects patients to high-risk conditions, using chemotherapies that cause temporary immunodeficiency and potentially life-threatening infections. Efforts are undertaken to try and achieve a subtler reprogramming of the unwanted immune reaction in a tissue-specific manner, thereby reducing risk of the procedure while maintaining its efficacy. As T1D is a heterogeneous disease, a (semi-) personalized approach is warranted for the selection of appropriate candidates and appropriate immune endpoints for these novel therapies. Detailed knowledge on the genetic background of affected individuals and their exposure to environmental risk modulators can be used to 'match' the patient to the correct treatment and reduce the unnecessary exposure of patients to therapies that are unlikely to provide them any benefit. Determining biomarkers that distinguish T1D patients from healthy individuals, genetic factors that can mediate environmental triggers to altered immune status and genetic variants that are causal for the association of T1D loci

with disease, as described in chapter 3, chapter 4 and chapter 5 of this thesis, will benefit patient selection and subsequent personalized treatment. Therefore, it is imperative that it remains a focus of attention alongside the development of novel therapeutic strategies. Currently several approaches using recombinant antibodies to immune-receptors, adoptive regulatory T cell therapy or vitamin D modified dendritic cells are in progress, and might prove efficacious and feasible options for the treatment of T1D. As with anything the proof of the pudding is in the eating, and results of these trials are eagerly awaited.

Other strategies for combatting T1D aim to restore the insulin deficit directly, and thereby glycemic control, in T1D, rather than correcting the autoimmune response. The development of automated, closed-loop systems that mimic the pancreas' ability to sense glucose and release insulin according to need promises improved glycaemic control and increasing quality-of-life for T1D patients. Although this provides great promise in terms of improved quality of life and reduces the chance of acute complications of insulin replacement therapy, the artificial pancreas is not expected to fully reach the accuracy of endogenous β cells and will probably not completely prevent the long-term complications of T1D. Advances in (islet) transplantation have made replacement of lost β cells a realistic option for T1D patients, although as with all transplantations this approach suffers greatly from the shortage of donor organs and side-effects associated with immunosuppressive drugs that are, ironically, toxic to β-cells. Furthermore, as autoimmunity has a long-lived memory, the disease often recurs, leaving patients again dependent on exogenous insulin administration. The development of islet-protecting encapsulations, that allows for the free transfer of insulin while preventing the destruction of transplanted islets, promises better graft survival and transplant success. Concurrently, new sources of β cells are being investigated and with recent breakthroughs in differentiation of embryonic stem cells to (neo) beta-cells and establishment of immortalized β cell lines renewable sources of β cells appear within reach.

With all of these approaches issues regarding safety, feasibility, efficacy and cost-effectiveness need to be addressed and the continuing efforts of all those involved in overcoming T1D will be needed for the years to come.

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