

Use of a systems biology approach to understand pancreatic β -cell death in Type 1 diabetes

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Abstract

Accumulating evidence indicates that β -cells die by apoptosis in T1DM (Type 1 diabetes mellitus). Apoptosis is an active gene-directed process, and recent observations suggest that β -cell apoptosis depends on the parallel and/or sequential up- and down-regulation of hundreds of genes controlled by key transcription factors such as NF- κ B (nuclear factor κ B) and STAT-1 (signal transducer and activator of transcription 1). Understanding the regulation of these gene networks, and how they modulate β -cell death and the 'dialogue' between β -cells and the immune system, will require a systems biology approach to the problem. This will hopefully allow the search for a cure for T1DM to move from a 'trial-and-error' approach to one that is really mechanistically driven.

Introduction

Pancreatic β -cells are the target of an autoimmune assault in T1DM (Type 1 diabetes mellitus), with progressive invasion of the islets by mononuclear cells and an inflammatory reaction termed insulinitis, leading to a loss of 70–80% of β -cells at the time of diagnosis [1]. β -Cell loss occurs slowly during the course of insulinitis, and accumulating evidence suggests that increased apoptosis is the cause of the gradual β -cell depletion in T1DM [2,3]. The slow destruction of β -cells, coupled with the autoimmune nature of the disease, suggests that T1DM is potentially preventable, opening the way for several intervention trials [4]. Unfortunately, the two major intervention trials, respectively based on the use of nicotinamide [ENDIT (European Nicotinamide Diabetes Intervention Trial)] [5] and insulin [DPT-1 (Diabetes Prevention Trial: Type 1)] [6] failed to prevent T1DM. In parallel, attempts to replace the lost β -cells have been undertaken by transplantation of islets isolated from brain-dead organ donors. The initial 1-year results of islet transplantation coupled to a steroid-free immunosuppressive regimen (the 'Edmonton Protocol') were very positive [7], but enthusiasm has decreased due to complications associated with the chronic immunosuppression and the gradual loss of islet function, with less than 30% of the patients remaining free of insulin therapy 2 years after transplantation [8–10]. A recently proposed approach to restore β -cell function in T1DM, namely induction of β -cell regeneration coupled to inhibition of autoimmunity [11,12], remains to be validated.

So, where are we now? On the positive side, we have learned that T1DM has an identifiable pre-clinical phase that provides the opportunity to translate innovative therapy into disease prevention, and that islet transplantation has the potential to reverse the disease after clinical onset. On the negative side, all attempts to halt β -cell loss in the face of an autoimmune assault have up to now failed, while islet transplantation faces several hurdles before it can become a clinical therapy. Against this background, what should we do to successfully prevent T1DM, or, once it is diagnosed, improve it by islet transplantation? These questions can be best answered by posing two additional questions: (i) do we understand well enough how β -cells are progressively killed by the immune system in T1DM to allow a targeted intervention to prevent β -cell loss?, and (ii) is the current research approach to the problem, based on a narrow focus on individual pathways, adequate? In our view, the present answer to both questions is, unfortunately, no. Why it is no and what we can do to change the picture is discussed below.

Gene networks regulating β -cell dysfunction and apoptosis in T1DM

Most of the research effort in the field of T1DM pathogenesis has focused on the immune system, with less attention paid to the pancreatic β -cells. The main form of β -cell death in the course of insulinitis is apoptosis, which is triggered by contact with activated macrophages and T-cells and exposure to soluble mediators secreted by these cells, such as cytokines, oxygen free radicals and NO (nitric oxide) [2,3]. When β -cells are exposed *in vitro* to the cytokine IL-1 β (interleukin 1 β), or to IL-1 β + IFN- γ (interferon γ), they present functional changes which are reminiscent of those observed in pre-diabetic patients, namely a preferential loss of the first-phase insulin release in response to glucose, probably caused by a decrease in the docking and fusion of insulin granules to the β -cell membrane [13], and a disproportionate increase in the proinsulin/insulin ratio [14]. Following prolonged

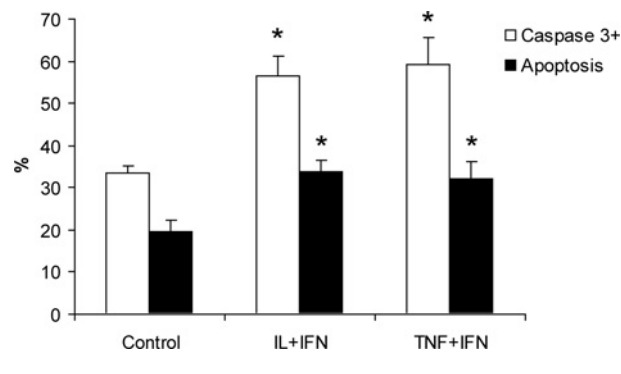
Key words: apoptosis, cytokine, diabetes mellitus, endoplasmic reticulum stress, pancreatic β -cell, systems biology.

Abbreviations used: dsRNA, double-stranded RNA; ER, endoplasmic reticulum; IFN- γ , interferon γ ; IL-1 β , interleukin 1 β ; iNOS, inducible NO synthase; IPA, Ingenuity Pathway Analysis; IRF-1, interferon regulatory factor 1; JAK, Janus kinase; MLDS, multiple low doses of streptozotocin; NF- κ B, nuclear factor κ B; NOD, non-obese diabetic; STAT-1, signal transducer and activator of transcription 1; T1DM, Type 1 diabetes mellitus; TNF α , tumour necrosis factor α .

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Figure 1 | Cytokine-induced apoptosis in purified rat β -cells

Rat β -cells were FACS-purified and exposed to IL-1 β (50 units/ml) + IFN- γ (100 units/ml) (IL + IFN) or TNF α (1000 units/ml) + IFN- γ (100 units/ml) (TNF + IFN) for 48 h [16]. Caspase 3 activation was determined by NucView™ 488 Caspase-3 Assay Kit for Live Cells (Biotium), while prevalence of apoptosis (%) was measured by the nuclear dyes Hoechst/propidium iodide [16]. Results are means \pm S.E.M. for five experiments; $P \leq 0.05$ compared with control by paired Student's t test. Similar findings were observed in insulin-producing INS-1E cells (not shown).



(2–7 days) exposure to IL-1 β + IFN- γ or to TNF α (tumour necrosis factor α) + IFN- γ , but not to either cytokine alone, β -cells undergo cell death mostly by apoptosis, with a minor necrotic component [2]. Under these conditions, caspase 3 activation precedes the nuclear changes that are characteristic of apoptosis (Figure 1).

Cytokines induce stress-response genes that either protect or contribute to β -cell death. They also down-regulate genes related to differentiated β -cell function and regeneration, and trigger the expression of chemokines and cytokines that will contribute to the attraction and activation of immune cells, leading to a ‘dialogue’ between the target β -cells and the invading immune cells [3,15]. There is, in parallel, an increased expression of MHC-related genes, which, coupled to cytokine-induced ER (endoplasmic reticulum) stress [16,17], may modify antigen expression and augment recognition of the β -cells by the immune system (Figures 2 and 3). Extensive microarray experiments by our group [15,18–21] have identified nearly 700 genes that are up- or down-regulated in purified rat β -cells or insulin-producing INS-1E cells after 1–24 h of exposure to the cytokines IL-1 β and/or IFN- γ , and nearly 2000 genes modified by cytokines or viral infection in human pancreatic islets [22]. The complete list of β -cell-expressed genes detected by the microarray analyses described above is freely accessible at the Beta Cell Gene Expression Bank (http://t1dbase.org/page/BCGB_Enter/display/) [23], developed by our group in collaboration with the Institute for Systems Biology (Seattle, WA, U.S.A.). The list of cytokine-modified rat genes (exposure time to cytokines of 24 h) [21] was introduced in the Ingenuity Pathway Analysis (IPA) software (version 5.0; <http://www.ingenuity.com>) to identify networks and pathways regulated by cytokines. The top four

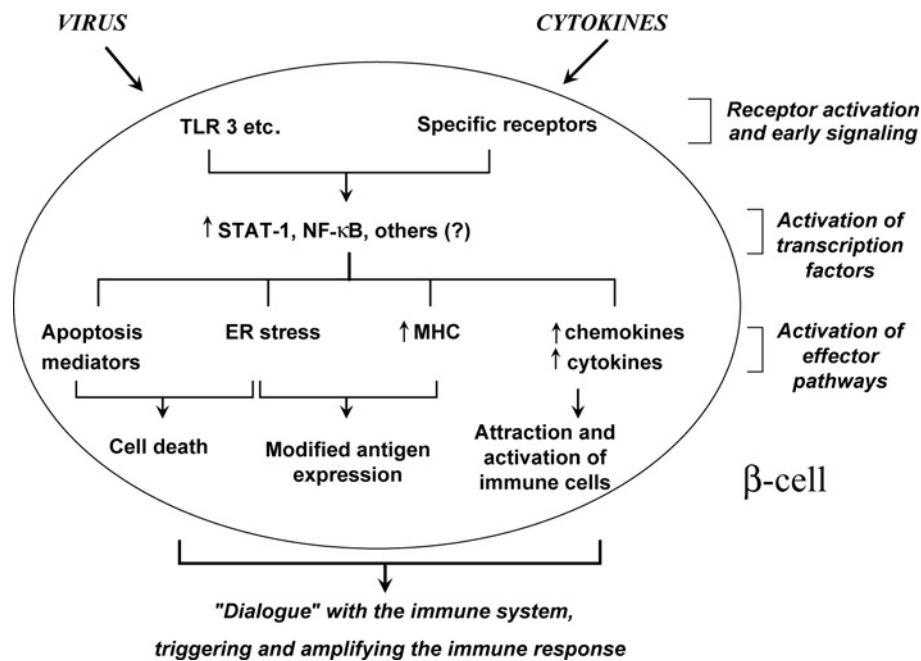
significantly changed canonical pathways included: ‘antigen presentation’ [15 genes, $-\log(P \text{ value}) = 9.054$], ‘Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2)-mediated oxidative stress response’ [29 genes, $-\log(P \text{ value}) = 5.678$], ‘interferon signalling’ [nine genes, $-\log(P \text{ value}) = 5.284$] and ‘NF- κ B (nuclear factor κ B) signalling’ [20 genes, $-\log(P \text{ value}) = 4.539$]. In the category functions, the highest P value was scored for ‘eukaryotic cell death’ [218 genes, $-\log(P \text{ value}) = 8.9 \times 10^{-26}$] with 18 genes allocated specifically to ‘cell death in pancreatic cells’. A total of 71 genes were allocated to the function of ‘immune response’ [$-\log(P \text{ value}) = 2.02 \times 10^{-8}$], with 23 genes associated specifically with ‘activation of antigen presenting cells’ [$-\log(P \text{ value}) = 1.04 \times 10^{-5}$]. A total of 28 networks with scores ≥ 10 and having more than 12 focus genes were identified (results not shown). These networks identify key transcription factors and their interaction modes. Similar categories were observed when analysing INS-1E cells exposed to cytokines for 12 h (results not shown).

IL-1 β or TNF- α alone do not induce apoptosis in purified human or rodent β -cells, but when these cytokines are combined with IFN- γ , approx. 30–50% of the β -cells undergo apoptosis after 2–9 days [2] (Figure 1). Two transcription factors play key roles for cytokine-induced apoptosis, namely NF- κ B (induced by IL-1 β /TNF α) and STAT-1 (signal transducer and activator of transcription-1) (induced by IFN- γ). In our IPA analysis, two networks containing STAT-1 were identified; these two were merged and are shown in Figure 3(A). Two networks contained the transcription factor NF- κ B; one of these networks also contained STAT-1. Figure 3(B) shows all of the genes regulated by NF- κ B.

IL-1 β and TNF α activate NF- κ B in β -cells, and array analysis of IL-1 β -treated β -cells whose NF- κ B activation was blocked [19], together with additional experiments [16,24], indicate that the transcription factor controls diverse gene networks that modulate the β -cell differentiated state and ER Ca²⁺ homeostasis, attract and activate immune cells, and contribute to β -cell apoptosis by both triggering NO synthesis and consequent ER stress [16,17] and by other mechanisms that remain to be determined [3,24]. NF- κ B is also induced in β -cells by extracellular dsRNA (double-stranded RNA) (a by-product of viral infection) [25], in a signalling pathway triggered by the activation of TLR3 (Toll-like receptor 3) [26]. Prevention of NF- κ B activation protects β -cells *in vitro* against IL-1 β + IFN- γ - [27–29] or dsRNA + IFN- γ -induced apoptosis [25], whereas *in vivo* conditional and β -cell-specific NF- κ B blocking prevents induction of diabetes by MLDS (multiple low doses of streptozotocin) [29], a treatment that induces a toxic/immuno-mediated diabetes with a strong component of islet inflammation [30]. Curiously, NF- κ B has mostly anti-apoptotic effects in other cell types [31], and recent results in NOD (non-obese diabetic) mice, an animal model of predominantly T-cell-mediated autoimmune diabetes, indicate that inhibition of β -cell NF- κ B activation by constitutive expression of a non-degradable form of I κ B α (inhibitor of NF- κ B α) accelerates the development of

Figure 2 | The 'dialogue' between β -cells and the immune system, which may lead to progressive β -cell death and amplification of the autoimmune assault

This process can be initiated by β -cell exposure to cytokines and/or viral infection, and will depend on receptor activation and early signalling, activation of key transcription factors (for instance, NF- κ B or STAT-1) and of effectors pathways of cell death (e.g. ER stress, mitochondrial pro-apoptotic signals). Release of chemokines and cytokines by the β -cells themselves may attract and activate additional infiltrating immune cells, aggravating insulinitis. Additional information and supporting references are provided in the text. TLR 3, Toll-like receptor 3.



diabetes [32]. Comparison between IL-1 β -induced NF- κ B in β -cells (where the transcription factor has a pro-apoptotic effects) and fibroblasts (where it has anti-apoptotic effect) shows that cytokine-induced NF- κ B activation in insulin-producing cells is more rapid, intense and sustained than in fibroblasts, leading to a more pronounced activation of the downstream genes for iNOS (inducible NO synthase), MCP-1 (monocyte chemoattractant protein 1) and Fas [24]. This indicates that the intensity of NF- κ B activation is important for the pro- or anti-apoptotic outcome, which is supported by the observation that transient and modest activation of NF- κ B by extracellular matrix favours preservation of β -cell function [33]. These findings suggest that NF- κ B-mediated anti- or pro-apoptotic effects *in vitro* are cell- and context-dependent. Activation of NF- κ B in β -cells *in vivo* may play a pro- or anti-apoptotic role, depending on the animal model of diabetes utilized (pro-apoptotic in the case of MLDS [29], anti-apoptotic in the case of NOD [32]) and possibly on the time window utilized for the NF- κ B inhibition (protection against diabetes in the case of temporary inactivation [29], aggravation of diabetes in the case of prolonged and constitutive inhibition [32]). Additional experiments, with conditional NF- κ B inhibition at different time windows during the process of insulinitis in NOD mice, are now required to clarify the role of NF- κ B in *in vivo* β -cell demise.

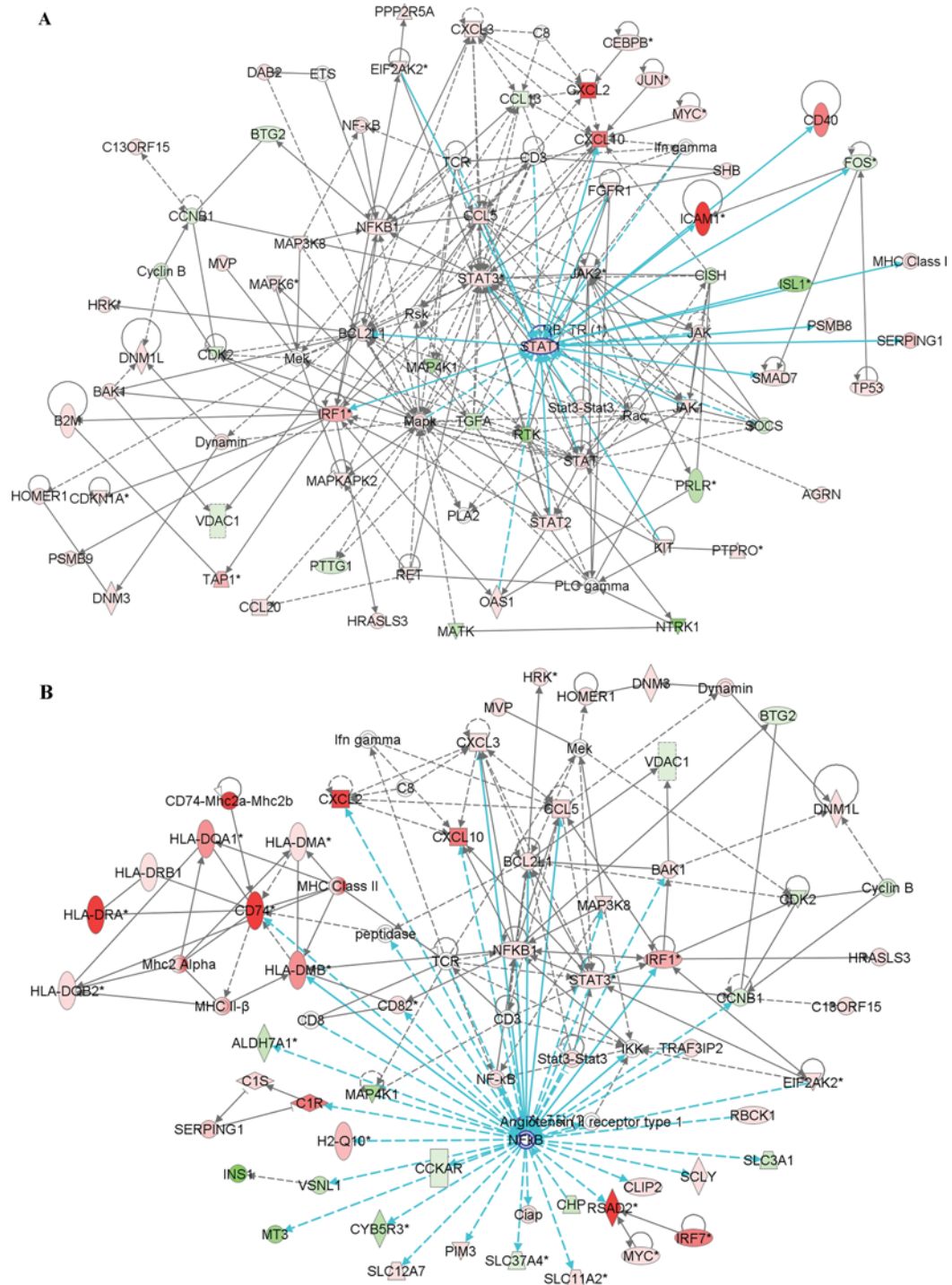
Following binding to cell-surface receptors, IFN- γ activates the tyrosine kinases JAK (Janus kinase) 1 and JAK2.

These kinases phosphorylate and activate the transcription factor STAT-1, which translocates to the nucleus and binds to γ -activated sites of diverse genes, such as the key transcription factor IRF-1 (interferon regulatory factor 1). STAT-1 mediates the potentiating effect of IFN- γ on IL-1 β -triggered iNOS gene expression [34], and islets and FACS-purified β -cells from STAT-1-deficient mice (STAT-1 $^{-/-}$) are protected against IL-1 β + IFN- γ -induced apoptosis [35]. β -Cells isolated from IRF-1 $^{-/-}$ mice are not protected against cytokine-induced β -cell death [36], suggesting that the IFN- γ pro-apoptotic signals are mediated by genes downstream of STAT-1, but not of IRF-1. The nature of these genes remains to be clarified, but some of them contribute to aggravate ER stress [37]. Systemic STAT-1 deletion protects against MLDS-induced diabetes [35] and spontaneous development of diabetes in NOD mice [38]. This protection depends in large part on interference with IFN- γ signal transduction at the β -cell level, as indicated by the fact that STAT-1 $^{-/-}$ islets transplanted into syngeneic wild-type mice protect them against MLDS-induced diabetes [39]. Transplantation of STAT-1 $^{-/-}$ islets into NOD mice causes less local inflammation, as compared with islet grafts from wild-type mice; although there is a lower prevalence of primary graft non-function with STAT-1 $^{-/-}$ islets, there is no protection against late islet graft rejection [39].

The concept of intervening in the process of immune-mediated β -cell destruction by acting at the target β -cell is

Figure 3 | Identification of STAT-1- and NF- κ B-regulated genes in INS-1E cells

The 700 significantly affected genes identified in INS-1E cells exposed to IL-1 β (10 units/ml) + IFN- γ (100 units/ml) for 24 h [21] were analysed with the IPA software. Genes with up-regulated expression (> 1.5) are coloured pink/red and genes with down-regulated expression (< -1.5) are coloured green. The intensity of the colour reflects the intensity of regulation after 24 h of cytokine treatment. **(A)** Two networks containing STAT-1-regulated genes were merged. Networks or connectivity maps contain genes regulated directly by STAT-1, which are shown in blue, whereas the nearest neighbour partners, as defined by the IPA connections, are shown in grey. For the highly regulated genes, we added representative IPA-suggested up- or down-regulation. Interestingly, these regulators were mainly regulating transcription factors (such as Myc and IRF7), autoantigens and complement factors. **(B)** Merged networks showing all genes regulated by NF- κ B. Two networks were regulated by NF- κ B, whereas one of these networks was co-regulated by STAT-1. Genes regulated by NF- κ B are highlighted in blue and the nearest neighbour partners, as defined by the IPA connections, are shown in grey.



supported by additional evidence. Thus: (i) overexpression of A20, a zinc-finger protein which inhibits NF- κ B activation, protects transplanted islets and decreases by 50% the number of islets required to attain normoglycaemia in diabetic rats [40]; (ii) adenovirus-mediated XIAP (X-linked inhibitor of apoptosis; a caspase inhibitor) gene expression increases human islet resistance against the deleterious effects of immunosuppressive drugs on viability and insulin secretion [41]; (iii) delivery of MnSOD (Mn²⁺ superoxide dismutase; a free radical scavenger) to mouse islets by a gene transfer approach improves islet survival after transplantation [42]; (iv) β -cells from ALR/Lt (alloxan-resistant) mice are protected against cytokine-induced β -cell death *in vitro* and NOD-derived diabetogenic T-cells *in vivo* based on their increased innate resistance to free radicals [43]; and (v) vitamin D analogues inhibit cytokine-induced chemokine and cytokine expression by islet cells, leading to a significant decrease in the severity of insulinitis and prevalence of diabetes in NOD mice [44].

The results described above indicate that the fate of β -cells after exposure to cytokines involves the perturbation of distinct β -cell gene networks regulated by NF- κ B, STAT-1 and other transcription factors that remain to be discovered. This suggests that the 'battle' leading to progressive β -cell loss in T1DM is fought, to a large extent, inside the β -cells, and leads to a 'dialogue' with the invading cells from the immune system that may amplify or dampen the immune assault (Figure 2). Depending on the relative contribution of intracellular β -cell events or the immune system to triggering diabetes in a specific animal model, intervening at the β -cell may suffice or not to prevent the disease. Thus blocking NF- κ B or STAT-1 protected against MLDS-induced diabetes [29,35,39], but failed to protect against the massive T-cell/macrophage assault leading to diabetes in NOD mice [32] or following islet transplantation [39]. On the basis of these observations, it can be speculated that prevention of human T1DM will require 'hitting multiple targets', i.e. preventing activation of pro-apoptotic β -cell gene networks, supporting β -cell defence/regeneration and arresting/regulating the autoimmune assault, which is a very difficult task that will require novel approaches to the problem. As suggested by the animal data, and taking into account that distinct animal models may represent particular 'subtypes' of human T1DM, the contribution of the different pathways involved may also vary between individual patients, indicating the need for individualized therapies. The concept of heterogeneity in the pathogenesis of human T1DM is supported by the absence of an hierarchy in autoantibody emergency in pre-diabetic individuals followed prospectively [45].

The need for a paradigm shift in diabetes research: the introduction of the systems biology approach

As discussed above, β -cell fate following exposure to immune mediators is a complex and highly regulated process, depending on the duration and severity of perturbation of key

interacting gene networks. This differs from the traditional view of biological phenomena, grounded in the characterization of signalling pathways by intuitive inferences based on the study of individual pathway components. Identification of complex and interacting gene/protein patterns was made possible by the sequencing of the human genome, and of the genome of several other species, and by the development of novel high-throughput technologies such as microarray analysis and proteomics. The use of massive parallel analysis of gene/protein expression fostered the concept that inter-pathway cross-talk reflects levels of complexity that cannot be explained by studying individual pathways in isolation. The full understanding of the cellular responses during the transition from physiology to pathology demands a global multivariate strategy, as proposed by the systems biology approach [46]. This approach aims to devise models based on the comprehensive qualitative and quantitative analysis of the diverse constituents of a cell or tissue, with the ultimate goal of explaining biological phenomena through the interaction of all its cellular and molecular components. This is based on the analysis of large-scale datasets, such as those generated by DNA microarrays and proteomics. The model is subsequently refined through introduction of perturbations in the system and new rounds of large scale gene/protein analysis. Systems biology is thus an interactive process in which researchers propose models based on large datasets, make predictions departing from the model, and then conduct additional large-scale experiments to test the prediction and refine the model [46]. This experimental strategy has been utilized in other cell types to study intracellular networks regulating cytokine-induced apoptosis [47] and the cellular responses to DNA damage [48]. We propose below a step-by-step approach, based on systems biology, to clarify the molecular mechanisms leading to β -cell death in T1DM.

1. Global evaluation of mRNA expression by microarray analysis and protein expression by proteomics of β -cells exposed to relevant pro-apoptotic stimuli, such as cytokines, dsRNA.
2. Identification of key gene networks and the transcription factors regulating them. Identification of these regulatory molecules can be based on both biological studies and *in silico* analysis (Figure 3) [49].
3. Introduce step-by-step perturbations in the system, preferentially targeting transcription factors with the potential of regulating key gene networks for cell death.
4. Evaluate whether these perturbations affect the biological end point, namely prevent or augment β -cell death.
5. Once this is achieved, perform new rounds of microarray and proteomic analyses to identify the genes and proteins downstream of the key transcription factors, and, based on this information, design novel interventions to modulate β -cell death. We have successfully followed this approach in the study of the transcription factor NF- κ B [19,24,27,29] and are presently utilizing it for the characterization of STAT-1 [35,39].

6. As the information accumulates, graphic display and digitalization of the biological output are made. To allow the research community to benefit from this information, the newly discovered genes and gene pathways are deposited in open access databases, such as the Beta Cell Gene Expression Bank at the T1Dbase [50].

By following this stepwise strategy, it will hopefully be possible to fully map the interacting networks of genes and proteins downstream of the pro-apoptotic signals leading to β -cell death and amplification of the immune assault. This will allow the search for a cure for T1DM to move from an empiric and blind approach to one that is really mechanistically driven; the ultimate outcome being the development of logical and targeted therapies to prevent the disease.

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