

Diabetes and stem cells according to: Matthias von Herrath and Dirk Homann

Islet regeneration needed for overcoming autoimmune destruction – considerations on the pathogenesis of type 1 diabetes

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Abstract: How many new β -cells need to be generated in order to withstand the attack of an ‘average-strength’ destructive autoimmune response? An answer to this question is central for the design of intervention approaches aimed at dampening or redirecting parts of the autoimmune response and allowing for the generation of new β -cells. In this article, we consider quantitative and spatial restrictions of destructive T-cell activity, in balance with the regenerative capacity and neogenesis of β -cells. We assume that the initial interaction between specific autoaggressive cytotoxic T-lymphocytes (CTL) and β -cells is a terminal event leading to the elimination of the β -cell and removal from the pool of potential sources for β -cell replenishment. Furthermore, we propose that there may be no way to save an individual islet from complete destruction, once a few activated CTL effectors have gained entry, based on the fact that activated CTL are ‘committed killers’ and hard to turn off. Thus, mechanisms that restrict CTL access to islets or provide ‘immune privilege’ to defined locations within islets and/or ductal tissue are critical to allow β -cell regeneration in the face of ongoing autoimmune destruction. The key to halting progression of type 1 diabetes pathogenesis should build on the observation that islets die in a highly non-synchronized fashion, at least during the more chronic disease course. These considerations suggest a compartmentalized view of the diseased pancreas so that substantial histopathological differences among individual islets may be exploited to facilitate preservation and/or regeneration of selected islets. The recent development of novel technologies will allow more precise quantification of *in vivo* destruction and regeneration in order to test these hypotheses.

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Type 1 diabetes – loss of β -cell mass is a continuous process, but ‘attack’ of individual islets is a discontinuous process

It is generally assumed that the loss of β -cells in type 1 diabetes occurs gradually over an extended period of time (1, 2). When the threshold from subclinical to symptomatic disease is crossed, 5–20% of β -cells are still alive (3). At this stage, insulin production is impaired by a variety of immunological and metabolic

mechanisms, yet not irreversibly. This phenomenon is of therapeutic importance, because euglycemia can, sometimes, be re-established following appropriate interventions even after the first onset of hyperglycemia (3, 4). In addition, decline of β -cell mass can be halted temporarily in recent-onset diabetics (and permanently in recent-onset, non-obese diabetic (NOD) mice), by administering non-Fc-binding anti-CD3 (4) and possibly hsp277 (5). Thus, the overall loss of β -cells in humans is a continuous process that results in clinically

overt hyperglycemia, when more than 90% of β -cell functionality has been lost. As β -cell functionality can apparently be regained over time after the administration of some immune interventions in recent-onset diabetics, β -cell loss appears, at least to a certain extent, reversible. There are three principal pathways by which total β -cell functionality may be enhanced – (i) reversion of impaired functionality in individual β -cells, (ii) self-duplication (replication) of β -cells, and (iii) stem cell differentiation (regeneration), resulting in increased β -cell mass (6). It is of note that a recent report by Dor et al. has provided compelling evidence that the regeneration of pre-existing β -cells, rather than pluripotent stem cells, is the dominant source for regeneration (7). How can the relatively steady loss of total β -cell mass be reconciled with the rather discrete and heterogeneous immunopathological events in the pancreas during chronic progressive diabetes in men and in mice, which leaves some islets completely destroyed, some heavily infiltrated, and some barely or not affected at all?

A possible explanation is that the entry of destructive T-cells into individual islets occurs as much by regulated extravasation events as by chance given a low number of specific T-cells with relevant autoaggressive functionality. In this way, new islets can become affected continuously, but the overall histological picture demonstrates islets at various stages of infiltration, as well as healthy and completely destroyed islets at any given time. Over time, this process will lead to the destruction of more and more islets, until lack of sufficient β -cell mass leads to clinical disease.

Because islets, once infiltrated, can, in principle, be destroyed rather rapidly (Fig. 1, left side), as is, e.g., the case after adoptive transfer of a larger number of autoaggressive T-cells (8), we assume that once a few activated aggressive T-cells have entered an islet (likely via high endothelial venules (HEV)), β -cells within this

particular islet will all progress toward death. In human type 1 diabetes, the demise of a single islet, as a function of a fewer number of destructive T-cells, will take longer, but may be completed within a few days to weeks (see next paragraph). In order to account for the slow clinical course of type 1 diabetes (autoantibodies and islet infiltration can precede clinical diabetes by several years), we estimate that new islets should only come under attack every month or so in human diabetes and every week or so in NOD experimental diabetes. Thus, the entry of autoaggressive T-cells into islets may be a rather rare and infrequent event. Under these circumstances, islet regeneration could play a significant part in influencing the course of disease, even if it occurs at a slow rate. Where and how rapidly will islet regeneration or replication of β -cells most likely occur in order to counterbalance the aggressive response? In order to find at least a theoretical answer to this problem, we need to consider how much of an impact the entry of a few autoaggressive T-cells will have on an individual islet.

How good is the autoaggressive response in eliminating β -cells? CD8 cytotoxic T-lymphocytes (CTL) are good killers

CTL are exquisite killers (9). Elegant microscopic studies have shown that morphological changes in target cells can be observed as soon as 5 min after exposure to CTL (10) and effector CTL can eliminate 10–40% of targets *in vivo* within 5 min (11, 12). Even ‘resting’ memory CTL can effectively kill targets *in vivo*, apparently as a clear function of the effector : target cell ratio (resulting in >80% of killing within 4 h at an effector : target ratio of 2 : 1 (12). Can similar kinetics be applied to the killing of β -cells? We believe that there is no strong reason not to do so, because β -cells can be killed readily *in vitro* by CTL, as long as they express the appropriate major histocompatibility complex (MHC):peptide complexes. MHC class-I on β -cells will increase even under mild conditions of inflammation, as demonstrated by several groups (13, 14). Although islets demonstrate a whole spectrum of sizes and are distributed unequally within the pancreas, it has been estimated that the ‘average’ islet contains about 200–300 β -cells. Therefore, a single aggressive CTL should, in principle, be capable of eliminating one islet within a few days. Taking into account the potential effects of counterregulatory and other cells, a ‘smooth’ killing process may be delayed, but it appears to be a reasonable estimate that an islet can be destroyed within a week, once a few activated, autoaggressive T-cells have gained entry. If the number of aggressive CTL is experimentally increased, the destruction of most islets occurs in a highly synchronized fashion and resulting clinical disease becomes apparent within a few days. This is shown, e.g., by

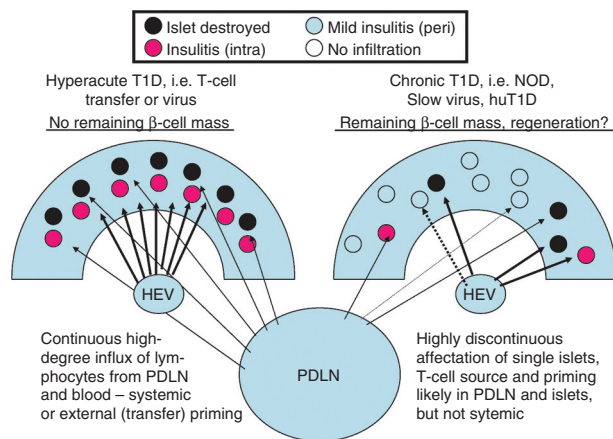


Fig. 1. In chronic type 1 diabetes (T1D), islet infiltration is not synchronized at all. In chronic but not hyperacute diabetes, islets are under various stages of attack. Non-obese diabetic (NOD); high endothelial venules (HEV); pancreatic draining lymph node (PDLN).

adoptive transfer of T-cells specific to an islet antigen (8) or the endogenous generation of a large number of CTL in the virus-induced diabetes rat insulin promoter-lymphocytic choriomeningitis virus (RIP-LCMV) model for rapid-onset diabetes (15) (Fig. 1, left side). Indeed, the latter model shows that the relative frequency of the original autoaggressors does not increase over time within islets and never exceeds more than 1/200 CD8 cells, and less than 1/400 CD8 cells in the more chronic lines (von Herrath, unpublished). Because histological analysis will only offer a 'snapshot', the heterogeneous picture under conditions of chronic disease could be because of the fact that islets are destroyed one by one in a slow progression toward clinical disease. If every islet remained infiltrated over a long time, one should see the infiltration of most islets during disease progression. Rather, kinetic histopathological analyses revealed a progressively declining number of unaffected islets in the presence of increasing 'islet scars' indicative of complete islet destruction. Moreover, if one considers the impressive efficiency of CTL killing, islets should rapidly succumb to the effects of CTL activation (i.e., perforin/granzyme-dependent killing), especially if coupled with the secretion of inflammatory cytokines, which will lead to further β -cell sensitization by the upregulation of MHC class-I expression (13).

Thus, even though 'insulinitis' at the level of an individual islet may not be chronic, the overall clinical course of the disease could still present itself in a chronic fashion because of discrete, stochastic yet, sequential, elimination of individual islets within the entire pancreas. What could 'drive' such recurring insults over an extended period of time? The pancreatic draining lymph node (PDLN) might be important as a local 'co-ordination center' that continuously provides the pancreas with destructive T-cells. Although their number is presumably very low, the aforementioned considerations suggest that these cells should at least, in theory, be capable of relatively rapid elimination of individual islets, which will cumulate in progressive destruction of the estimated 200–300 islets in the NOD pancreas and clinical disease within approximately 4 months. Given the higher number of islets in human pancreata, this process should take correspondingly longer. If the gradually increasing damage is, indeed, performed by a rather small number of CTL, their detection *in vivo* might be almost impossible in the periphery, unless one looks directly in the target organ, an endeavor that has now been undertaken by several research groups. Antigens shuttled to the PDLN could drive such a chronic process, even if they are presented only by a few antigen-presenting cells and, thus, also hard to identify.

What could limit the process? Probably regulation will rarely occur within the infiltrate of an islet, because CTL, once they have been committed as

activated effectors, cannot be switched off, at least not easily. In addition, we proposed that the large number of T- and B-cells seen in murine models within islets that are completely infiltrated might actually be a 'bystander aftermath' of a highly antigen-specific process that occurred earlier, involving only a few CTL. In support of this argument is the histological finding that fully infiltrated islets (profound intra-islet insulinitis) only contain very few to no remaining β -cells. Autoreactive regulatory cells could arise later during the infiltration and then circulate back to the PDLN (rather than the islet), where they are known to act as 'bystander suppressors'. It follows that the crucial limiting factors in islet destruction could be:

- Entry of aggressive T-cells into islets.
- Co-ordination and counterregulation of a steady supply of activated autoaggressive CTL in the PDLN. The only alternative is that activated CTL can migrate from islet to islet, which is rather unlikely, because they would have to be enticed to transmigrate through non-inflamed exocrine pancreatic tissue.

Last, it is important to explain the frequently observed histological phenomenon of periinsulinitis. Two factors may account for this phenomenon. First, pronounced infiltration of lymphocytes has been observed in animal models, where inflammatory cytokines were expressed in islets (i.e., interleukin-12 (IL-12) or IL-2) (16). In these cases, no β -cell destruction occurred and no islet antigen-specific T-cells were found within the periinsulinitic infiltrate. We believe that lymphocytes will be forced to the periphery of the islets because of anatomic constraints, if no antigen-specific destruction of β -cells that disrupt islet integrity occurs. Second, periinsulinitis has been described in situations of effective immune regulation. Although regulatory T-cells might result in relatively fewer islets being affected, the precise effect of bystander suppression on T-cell migration, e.g., is not yet known. It is doubtful that periinsulinitis occurs in the presence of many activated antigen-specific lymphocytes. Periinsulinitis might, thus, be a remnant of aggressive insulinitis in mice, where new autoaggressors are not generated anymore in the PDLN. Under conditions of bystander suppression, e.g., the islets that were under attack have already been, for the most part, destroyed, and a sufficient number of non-affected islets were left to maintain normoglycemia. These simple kinetic considerations could help us explain the histopathology observed in type 1 diabetes models and may assist in refocusing our attention on lymphocyte entry into the islets and counterregulation in the PDLN. One last area that needs to be discussed in the context of our hypothesis is islet regeneration.

How good is the regenerative response?

As mentioned before, there are three principal pathways to reinvigorate β -cell function at the population level – functional rescue, β -cell replication, and differentiation from precursor cells (6, 17, 18). The differentiation process has been observed in chimeric mice, as well as in pancreatic ducts, and might provide an immune privilege for the newly generated β -cells (19, 20). However, recent findings suggest that β -cell replication may be a particularly important source (7). The last two processes may also be driven by relative insulin insufficiency, as is the case in obese people, pregnancy, type 2, and type 1 diabetes (21). Because β -cell replication within an islet would have to be extraordinarily fast to counteract CTL killing within a given islet, we presume that this process will be insufficient to rescue an islet already under attack. Thus, increased β -cell replication in unaffected islets will be of greater importance, because such newly regenerated β -cells or islets would probably experience some immune privilege, in the sense that they have not yet been infiltrated by CTL and can grow ‘in peace’, at least until aggressive T-cells reach them for the first time. We conclude that β -cell regeneration, as well as β -cell replication, can be important in preventing hyperglycemia in pre-diabetic people, but, only if they occur in anatomically protected areas where β -cells are not immediately subject to attack by aggressive T-cells. Indeed, if one considers the kinetic circumstances of a regular *in vitro* cytotoxic T-cell assay, the live fibroblasts that usually function as target cells are never capable of ‘outgrowing’ the direct attack, although they are relatively rapidly dividing cells. Thus, treatments relying on β -cell regeneration and/or replication should be accompanied by a strong, temporary suppression of the aggressive response.

Conclusions

Which are the conclusions that can be drawn, if our quantitative considerations are correct, and which are the crucial experiments that need to be performed in order to validate missing elements of our propositions?

- We believe that *in vivo* imaging should be used in order to study the precise point and overall process of the entry of activated autoaggressors into islets. Such an investigation could also assess how many β -cells a CTL can kill *in vivo* within a given time frame, when an activated CTL dies *in vivo*, and whether autoaggressive effectors can migrate from one islet to another via the exocrine pancreas. If CTL kill *in vivo* as rapidly as they do *in vitro*, it follows that an islet is essentially lost as soon as some CTL specific to islet antigens have entered; β -cell replication within this islet will be of no

effect (β -cells are highly unlikely to divide every 30 min!). Second, if CTL do not migrate from islet to islet, which is to be expected, because the exocrine pancreas is usually not inflamed, each affected islet constitutes a rather independent inflammatory process. Released β -cell antigens will, therefore, turn up mostly in the pancreatic lymph node.

- We have to understand whether or not antigenic spreading has any role in the aggressive response within an islet. Our hypothesis is that it will not, which still allows for the possibility that aggressive T-cells, with new specificities, can become activated within the PDLN and attack new islets from there. By contrast, based on the findings by many laboratories, antigenic spreading is likely of essence to propagate autoantigen-specific regulators that can act as bystander suppressors.
- It would be good to better understand the role of the PDLN as a co-ordinative center that generates a small, but steady, number of autoaggressive CTL over time. Based on our hypothesis, β -cell antigens will be regularly presented there, once islets have come under attack, but activated autoaggressors might only relatively rarely emerge from the PDLN and reach the islets. Indeed, in order to get there, they will have to go the systemic route via the blood and enter the islets through the HEV. This will be a stochastic event, unless islets are already inflamed and attract such CTL with chemokines. There is strong evidence that chemokines generated after viral infection of the pancreas can be instrumental in attracting autoaggressive T-cells to the islets. *In vivo* imaging could shine more light on the role of the PDLN and the amount of traffic between the PDLN and the islets.
- We should better understand the phenomenon of periinsulinitis. Our hypothesis is that this occurs in the absence of antigen-specific aggressive T-cells, when β -cell and islet integrity is largely preserved, but inflammatory cytokines are present. Following interventions, periinsulinitis might remain in those cases where the few autoaggressive driver clones were recruited away from the islet.

If our hypothesis is correct, the essential factors determining the pathogenesis of type 1 diabetes are the entry of a few autoaggressors into single islets, the chronic generation of a steady supply of such aggressive CTL, the regeneration of β -cells outside the islets, and the driver antigen(s) and T-cells. It follows that the fate of an islet might be sealed long before profound infiltration is seen, which is mainly composed of antigen-non-specific bystander cells. Therapy, therefore, should focus on the issues of trafficking, islet regeneration, and modulation of immunity in the PDLN as the

site of islet antigen presentation, but not the islets themselves. Islet regeneration could be relatively a slow process to maintain normoglycemia, if it occurs outside attacked islets. By contrast, β -cell replication within an islet that contains a few differentiated and activated effector CTL would have to take place extremely efficaciously to make any difference.

It is striking to observe that during more chronic diabetes development, as it, e.g., occurs in NOD or RIP-LCMV-nucleoprotein (NP) mice, not all islets are at the same stage of infiltration (Fig. 1, right side). This also applies to most cases of human type 1 diabetes, as far as we know from the more limited amount of pre-diabetic pancreata available. Some islets usually exhibit no infiltration at all (these might be the main source for the remaining β -cell mass present at the onset of hyperglycemia in humans and slow onset in mouse models). Some islets show only mild insulinitis, mainly found around the periphery, and some show more severe insulinitis. Islets that have been completely destroyed have no infiltration and only some scarring is evident. However, we have learned from animal models with rapid diabetes (within 7 days, e.g., following adoptive transfer of a high number of aggressive CD4 and CD8 T-cells) that islet destruction can, in principle, occur very rapidly and in a highly synchronized fashion (Fig. 1, left side). In these models, divergent stages of infiltration are less evident. What could these observations tell us? They allow for the conclusion that slowly progressive, chronic diabetes might not be characterized by a drastically different type of infiltration, but rather by the fact that autoaggressive T-cells gain entry to islets less frequently. Possibly, once a sufficient amount of aggressive T-cells has entered a given islet, the fate of this particular islet is sealed and destruction will result rather rapidly, maybe as fast as in a few days. If this is true – absolute validation, unfortunately, can only be achieved by means of long-term *in vivo* imaging of islets and the infiltrative process – the main determinant in the pathogenesis is the generation and the number of autoaggressive T-cells and their ability to gain access to non-inflamed, ‘naïve’ islets. We do not know, at this point, which factors precisely control the entry of naive T-cells into islets. Most likely, they have to transmigrate into the tissue via HEV, unless it is possible that activated autoaggressors can migrate from islet to islet through the exocrine pancreatic tissue. Clearly, activated autoaggressors will gain entry into tissue much more easily and chemokines are key candidates in attracting such cells. Thus, in chronic diabetes, some of such activated cells will have to be supplied from time to time. Based on our quantitative considerations presented in this article, this can be a rather infrequent event. A likely source is the PDLN, where islet autoantigens can be (cross)-presented in a proper lymphoid context.

Based on these thoughts, the following issues become the most pertinent to resolving and understanding the pathogenesis of type 1 diabetes:

- Once a few activated CD8 CTL have entered an islet, is the ensuing destructive process determined? Is a significant influx of new autoaggressive CTL necessary?
- How long will it take for the single islet to ‘fall’? Quantitative considerations presented in this article indicate that this could happen within a few days.
- Where does islet regeneration come into play? It might be unlikely that β -cells within a given islet ‘under attack’ can regenerate as rapidly as CTL can eliminate them. Thus, regeneration from stem cells or at privileged sites – i.e., in the ducts – might be important.
- For new islets to come under attack, can activated CTL come from other islets or do they come from the PDLN and/or periphery?
- How many β -cells can one activated CTL kill *in vivo* during its life?

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