Thymus-specific deletion of insulin induces autoimmune diabetes

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Review timeline:

- Submission date: 25 March 2009
- Editorial Decision: 27 April 2009
- Revision received: 05 June 2009
- Editorial Decision: 26 June 2009
- Revision received: 30 June 2009
- Accepted: 01 July 2009

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 April 2009

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees. As you can see below and as is also indicated in the comments to the editor, the referees find the analysis interesting and suitable for the Journal. However, the referees also do bring up some specific issues, as detailed below, that would need to be resolved. Given these comments I would therefore like to invite you to submit a suitably revised manuscript that takes these issues into account.

I thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This interesting paper addresses an old, yet never formally analyzed question: it demonstrates, in a definitive fashion, the critical role of thymic self antigen expression, using type 1 diabetes as a model for spontaneous autoimmunity. The technical approaches are elegant and well designed. There are few issues that should be addressed:
1. The authors should comment on whether the transplant of ID-TEC thymus to nude mice actually results in diabetes and not just insulin autoreactivity. If hyperglycemia does not occur, then the diabetes seen in ID-TEC mice is not just a central tolerance phenomenon but involves some definite contribution of the transgene in the periphery.

2. Can immunotherapy with insulin prevent or delay the diabetes in ID-TEC mice?

3. Error in the intro. The insulin transgene replaces native tyrosine with alanine (not vice versa as stated)

4. Authors should comment on the band seen in the pancreas of Fig 3A. That is, Cre transcripts are observed in the pancreas.

5. Figure legend for figure 3 is incorrectly labeled. A description of D is cited as E.

6. There are typographical errors in figure legend of Fig6C.

Referee #2 (Remarks to the Author):

In this manuscript, the authors develop a novel mouse model of type-I insulin dependent diabetes (T1D), and provide data supporting the hypothesis that thymic expression of insulin is itself essential for prevention of T1D. Previous work has established that insulin serves as a significant autoantigen that provokes T1D in both human disease and in mouse models such as the NOD mouse. What is novel and somewhat surprising is that thymus restricted expression of insulin is required for deletion of insulin-specific autoreactive T cells, cells that eventually migrate into the pancreas and mediate the destruction of insulin-producing beta cells. Indeed, as the authors suggest in their discussion, equally likely is that circulating insulin would be collected, processed and expressed by thymic antigen presenting cells. The authors present compelling evidence that insulin is expressed by thymic epithelial cells (mTEC), a cell type known to induce negative selection by presenting self-antigens. Of these self-antigens, a variety of tissue and stage specific antigens not normally associated with thymic function are expressed in mTECs by virtue of the high level expression of the transcription factor Aire. Thus, during T cell development in the thymus, those T cells capable of binding with high avidity to mTECs expressing such peripheral tissue antigens (PTAs) are deleted by negative selection.

To address the potential that insulin expression in the thymus (by virtue of Aire function) leads to deletion of diabetogenic T cells, the authors have developed a line of mice (called ID-TEC) that lack insulin specifically in these mTEC. For this, the authors generated insulin-2 floxed mice that were then bred with insulin1/-/- mice. As well, the authors generated a novel Cre expressing mouse that will likely be useful as a tool for many other studies. In this mouse, Cre expression is mediated by the Aire promoter, thus leading to highly specific expression of functional Cre (as assessed using the Rosa26LacZ mouse strain) in mTECs. Once bred with the Ins1/-/- x Ins2l/l mice, the authors demonstrated specific deletion of Ins2 in the mTEC population in the thymus. With this, the result was the development of acute hallmarks of T1D, including hyperglycemia and insulitis in very young mice. Transfer of CD4 or CD8 T cells from ID-TEC mice to Rag/-/- mice led to some (but not all) hallmarks of T1D, demonstrating that the disease was due to autoreactive T cells. Overall, the results are extremely compelling and the findings of significance to those studying autoimmunity and T cell tolerance. With one exception, the concerns are relatively minor.

Major concern: while the authors contend that deletion of both insulin isoforms in mTECs prevents deletion of insulin-specific T cells, an alternative explanation is that such mice may have a more generalized defect in negative selection. Although this is unlikely, it is possible that mTEC-dependent insulin expression may have a role in these mTECs or other thymus cells. To address this contention, the authors should demonstrate that negative selection to other "auto-antigens" is unimpaired in the ID-TEC mice. This could be done by breeding the mice with TCR-transgenic animals (e.g. male H-Y specific, etc), or by evaluating deletion of V-beta T cell subsets by endogenous superantigens. Again, while this is an unlikely scenario, defective antigen presentation or function during thymopoiesis could certainly lead to defective negative selection.
Minor concerns:
1. The manuscript requires a careful review for typographical and lexical errors. I noted several, including in figure legends with numbers non-existent in figures. Also, I noted the use of "residue" when the word "residual" would be more appropriate. Additionally, more effective labeling of the figure panels themselves would be of benefit to the readers.
2. The issue regarding regulatory cells is somewhat confusing the way it is described in the last section of the Results. The authors demonstrate that Treg from ID-TEC mice function normally, an important control. However, they state that they believe the more modest T1D hallmarks observed after T cell adoptive transfer might be due to "suppressive activities." Since they are apparently transferring anti-CD25 depleted T cells into Rag-deficient hosts, one would assume that the disease would be more profound. This should be clarified, and/or simplified. They could probably just get away with mentioning that they looked at Treg function and found it to be unimpaired in the ID-TEC mice. How this might affect the adoptive transfer experiments seems a bit of a stretch.
3. In Fig. 4, the authors present data demonstrating near complete loss of insulin mRNA in ID-TEC cells by RT-PCR analysis. However, they don't state which insulin isoform they are assaying here. Most likely, this is insulin-2, but they need to be more specific about this. In this regard, it is somewhat surprising that deletion of insulin in mTEC leads to such rapid deletion and loss of insulin peptides presented by these cells. How do the authors account for such rapid deletion and loss of insulin presentation (or does the deletion take place at an earlier stage in the development of mTEC)? This should be addressed in the discussion.

Referee #3 (Remarks to the Author):

This manuscript describes a surprising outcome of an experiment many people would be surprised has been carried out. It has been known for a long time that genetic deficiency of a protein allows immune responses to be made to that protein - this has been extensively exploited in generation of monoclonal antibodies. Such mice do not generally develop spontaneous autoimmune disease because of course they lack the protein in the target tissue. In the case of insulin this is complicated by the existence of two closely related insulin genes in mice. Generalized deletion of the mouse insulin 2 gene was reported to accelerate diabetes in diabetes-prone NOD mice in 2003 by Boitard and colleagues whereas deletion of the insulin 1 gene reduced autoimmunity and diabetes (described by Eisenbarth and colleagues). Insulin 2 deficient mice on the non-autoimmune prone 129 strain did not develop autoimmunity spontaneously but could be immunized with insulin peptides. These and other studies indicated that deletion of insulin 2 enhanced autoimmunity and established thymic (or "central") immune tolerance to insulin as a vital protective mechanism against the development of diabetes.

The current study significantly refines this idea by deleting the insulin 2 gene only in thymic epithelial cells, leaving insulin 2 gene expression intact in the pancreatic islets. This is the only insulin present because insulin 1 knockouts were bred with the insulin 2 conditional knockouts. These mice had the surprising finding of developing highly penetrant diabetes at a young age. MHC genes are by far the major risk allele for the development of diabetes however the mechanism by which some MHC alleles protect and some predispose is poorly understood. The mice in this study developed diabetes despite carrying the "protective" H-2b haplotype. This suggests that the protection endowed by H-2b depends on the presence of insulin -most probably because insulin peptides presented by H-2b induce immune tolerance in normal mice. The corollary perhaps is that normally predisposing MHC types such as 1-Ag7 in mice or HLA-DQ8 in man may be deficient in the ability to delete autoreactive T cells specific for insulin. This result - that of turning H-2 b from protective to permissive by deletion of insulin seems to be a major insight and a novel discovery. Despite this the paper has some loose ends:

- The histology of the pancreas is quite incompletely characterized - there is a focus on CD4 T cells only and it would be good to see staining for CD8 T cells, B cells, macrophages etc.
- The most robust test for insulin autoimmunity at present is insulin autoantibodies (IAA). It would be important to show data on IAA in the mice as well as the islet autoantibody assay that is shown.
- The assays for T cell responses to autoantigens appear to show responses to insulin but not GAD. However, a GAD peptide, GAD 206-220, defined in NOD mice was used to test for GAD responses. Given the different MHC type of the NOD and B6 mice it is not obvious
why H-2b mice would respond to this peptide. Whole GAD or GAD peptides known to be presented by H-2b would be a much better control.

- Concerns linger about the studies involving cell transfer and thymus transplant that have been done to confirm the autoimmune nature of the diabetes. In neither case was transfer of diabetes fully achieved although histological abnormalities were certainly present. These results raise questions about whether the presence of insulin 1 deficiency is important in developing diabetes. It would be good to see data shown on autoimmunity in mice that have thymic deletion of ins2 but ins1 is intact - clearly these data would have been generated in the course of the studies.

- The T cell responses to insulin could be further developed to examine the epitopes of insulin that are responsible. This could be done by replacing intact insulin with panels of insulin peptides in the interferon-gamma ELISA test shown in Fig 6E.

Referee #1

1. The author should comment on whether the transplant of ID-TEC thymus to nude mice actually results in diabetes and not just insulin autoreactivity. If hyperglycemia does not occur, then the diabetes seen in ID-TEC mice is not just a central tolerance phenomenon but involves some definite contribution of the transgene in the periphery.

*We added the following comments to the discussion (Page 19, 2nd paragraph), giving a number of potential explanations on the lack of full-blown clinical diabetes in our transfer experiments:

"Neither our thymus-transplantation nor T-cell adoptive transfer experiment recapitulated the full-blown clinical diabetes seen in ID-TEC pups, although islet-specific autoimmunity was evident. In both cases, the T-cells transferred or migrating from the transplanted ID-TEC thymus would expand in the lymphopenic environment of an immune compromised adult mouse, a situation clearly different from that of a newborn pup (Garcia et al, 2000). In addition, studies of neonatal mice showed that the development of effector T-cells precede that of Treg cells (Monteiro et al, 2008). Within the neonatal environment, establishment of proper Teff/Treg ratio is crucial to prevent autoimmune disease later in adult life. Indeed, delay of Treg migration from the thymus via thymectomy at day 3, has been associated with organ-specific autoimmune disease (Sakaguchi 2004). Without negative selection of insulin-specific T-cells, the initial wave of effector T-cells seeding the periphery of ID-TEC pups would contain a substantial higher number of insulin-specific T-cells, which could disturb the proper ratio of Teff/Treg cells. This unchecked expansion of insulin-reactive effector T-cells could inflict irreversible damage to the -cells prior to the expansion of Treg cells to establish tolerance. We are currently generating insulin-specific T-cell clones from ID-TEC mice to test this possibility. Another unique feature in neonatal pups is the wave of excessive -cell apoptosis during the second and the third week of postnatal life (Scaglia et al, 1997; Trudeau et al, 2000). The shedding of islet antigens through the apoptotic process was able to activate antigen presenting cells and initiated insulitis in prediabetic NOD mice. Since a high-level expression of the Aire-Cre transgene was observed in the newborn ID-TEC thymus (our unpublished observations*), negative selection of insulin-specific thymocytes could be compromised at birth due to the Cre-mediated abrogation of insulin expression. Although neither insulitis nor loss of insulin content was observed in ID-TEC pancreas at day 10, elevated blood sugar levels were observed in the third postnatal week, when signs of islet inflammation are present. Thus, -cell apoptosis might play an indispensable role in initiating and amplifying the islet-specific autoimmune response in ID-TEC mice, which was absent in transplantation models using adult immune compromised mice as recipients. Alternatively but not mutually exclusively of the above mechanisms, other peripheral self-antigen tolerance mechanisms could limit the progression of islet autoimmune destruction."

* These data are shown in referee-only Figure R1.

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2. Can immunotherapy with insulin prevent or delay the diabetes in ID-TEC mice?

Our study presented in this paper focused on the etiology of type 1 diabetes and investigated the functional importance of thymic insulin expression in regulating immune tolerance towards insulin-secreting pancreatic β-cells, even in the context of diabetes-resistant MHC. The interaction between peripheral tolerance mechanisms and central negative selection is certainly of great interest to us, and we believe that ID-TEC mouse could serve as a good model for the study. Like the reviewer, we are also curious about whether immunotherapy with insulin (induction of peripheral tolerance) can suppress or limit the pathologic autoimmune response of insulin-specific autoreactive T-cells towards pancreatic β-cells, and are currently working in this direction. However, we believe this study is out of the scope of this paper.

3. Error in the intro. The insulin transgene replaces naïve tyrosine with alanine (not vice versa as stated).

We thank the reviewer for pointing out the error to us, and corrected it accordingly (Page 4, paragraph 1).

4. Authors should comment on the band seen in the pancreas of Fig 3A. That is Cre transcripts are observed in the pancreas.

Considering the referee’s comment, we acknowledged in the text that there is a faint Cre band in the pancreas by modifying the following sentence (Page 7, paragraph 3): "As shown, the Aire-Cre transgene was predominantly expressed in the thymus, albeit weak Cre signals were detected in other organs, such as the spleen, the kidney and the pancreas.” However, this weak band is unlikely from pancreatic beta cells (which is the major concern) as Cre transcript is absent in the isolated islets. We then added the sentences: "We also examined the presence of Cre transcript in isolated pancreatic islets (IS), to rule out the possibility that the faint Cre-band observed in the whole pancreas tissue was from islet β-cells. In the purified β-cells, no Cre transcript was observed (Figure 3B).” (Page 8, paragraph 1).

5. Figure legend for figure 3 is incorrectly labeled. A description of D is cited as E.

Figure legend for figure 3 is corrected. We apologize for the oversight in our initial submission.

6. There are typographical errors in figure legend of Fig6C.

Figure legend was corrected. Again, we apologize for our oversight.

Referee #2

Major Concern: while the authors contend that deletion of both insulin isoforms in mTECs prevents deletion of insulin-specific T cells, an alternative explanation is that such mice may have a more generalized defect in negative selection. Although this is unlikely, it is possible that mTEC-dependent insulin expression may have a role in these mTEC or other cells in the thymus. To address this contention, the authors should demonstrate that negative selection to other "auto-antigens” is unimpaired in the ID-TEC mice. This could be done by breeding the mice with TCR-transgenic animals (e.g. male H-Y specific, etc), or by evaluating deletion of V-beta T cell subsets by endogenous superantigens. Again, while this is an unlikely scenario, defective antigen presentation or function during thymopoiesis could certainly lead to defective negative selection.

We fully agree with the referee on the importance of ruling out the alternative, yet rather “unlikely” scenario in which the lack of insulin expression in the medulla of the thymus could cause a more generalized defect and compromise the overall negative selection process towards auto-antigens. We thank the referee for recommending two general directions, which helped us to refine our approaches to address this issue.

Since ID-TEC mouse carries three genetic modifications: both the Ins2-floxed and the Ins1-null alleles were fixed as homozygous, whereas the Aire-Cre transgene was hemizygous, introducing a
TCR-transgene to the model would require a minimum of three rounds of breeding, a good but impractical approach to meet the 90-day deadline of the reviewing process. Thus, to address this issue and to exclude any generalized impairment of negative selection in ID-TEC mice, we used H2b-restricted, male-specific HY antigens as model autoantigens, as suggested by the reviewer. In this model, autoreactive thymocytes specific to HY antigens are eliminated in the male thymus, but are not negatively selected in the female thymus due to the absence of male antigen expression/presentation. Consequently, if the negative selection works normally, HY autoantigen-specific T-cells should be absent in the periphery of male mouse, but can be readily detected in females upon HY autoantigen challenge. Examination of the presence of T-cells specific to the HY autoantigens enabled us to confirm the overall efficacy of thymic negative selection in ID-TEC mice.

We presented the results of these experiments in Figure 9 (A-C, page 14). As shown in Figure 9B, male autoantigen Uty-specific CD8+ T-cells are essentially absent in male splenocytes, indicating efficient negative selection driven by the autoantigens in the thymi of both ID-TEC and control males. In contrast, CD8+ T-cells specific for male antigen Uty are readily detectable in ID-TEC females by Db/Uty tetramers following sensitization with male splenocytes, demonstrating the intact ability to elicit an immune response upon antigen challenge. Additionally, MHC class II molecule mediated negative selection of CD4+ thymocytes was evaluated by ELISPOT assay, using I-Aβ-restricted HY antigen Dby as stimulant for IFN-γ secretion. As shown in Figure 9C, responding cells were present only in spleens of either ID-TEC or control females, but absent in ID-TEC or control males. Thus, abrogation of insulin expression in mTEC did not cause any generalized impairment in the negative selection of autoreactive T-cells.

Minor Concerns:

1. The manuscript requires a careful review for typographical and lexical errors. I noted several, including in figure legends with numbers non-existent in figures. Also, I noted the use of residue when the word residual would be more appropriate. Additionally, more effectively labeling of the figure panels themselves would be of benefit to the readers.

We thank the reviewer for pointing out the errors and apologize for the oversight in our initial submission. We carefully examined the manuscript and corrected all the typographical and lexical errors in the text and figure legends. In addition, we reorganized the figure panels in Figure 1, 3 and 5 to make them more legible to readers.

2. The issue regarding regulatory cells is somewhat confusing the way it is described in the last section of the Results. The authors demonstrate that Treg from ID-TEC mice function normally, an important control. However, they state that they believe the more modest T1D hallmarks observed after T cell adoptive transfer might be due to “suppressive activities.” Since they are apparently transferring anti-CD25 depleted T cells into Rag-deficient hosts, one would assume that the disease would be more profound. This should be clarified, and/or simplified. They could probably just get away with mentioning that they looked at Treg function and found it to be unimpaired in the ID-TEC mice. How this might affect the adoptive transfer experiments seems a bit of a stretch.

We significantly reduced and simplified the arguments relevant to Treg cells in both the Results and the Discussion sections of the paper. In the Results, we only pointed out the facts that there is no overall numeric change in Treg cells of ID-TEC mice, compared to littermate controls; and that their suppressive function to control anti-islet autoimmunity is retained in ID-TEC mice (Page 15). In the Discussion, we restructure the paragraph (Page 19, paragraph 2), limiting the discussion to potential explanations.

3. In Fig. 4, the authors present data demonstrating near complete loss of insulin mRNA in ID-TEC cells by RT-PCR analysis. However, they donot state which insulin isoform they are assaying here. Most likely, this is insulin-2, but they need to be more specific about this. In this regard, it is somewhat surprising that deletion of insulin in mTEC leads to such rapid deletion and loss of insulin peptides presented by these cells. How do the authors account for such rapid deletion and loss of insulin presentation (or does the deletion take place at an earlier stage in the development of mTEC)? This should be addressed in the discussion.

The primer pairs we used to detect insulin mRNA in ID-TEC are specific for Ins2. Since the whole
Ins1 genomic region was replaced with the Neo cassette in the Ins1 knockout mouse, there is no Ins1 transcript in ID-TEC mouse. Nevertheless, we changed "insulin" expression to "Ins2" expression for clarification (Page 9, paragraph 3). We also examined Aire-Cre expression in thymi of neonatal pups (day 1) and readily detected Cre transcripts in the thymi of Aire-Cre transgenic pups (referee only Figure R1), suggesting that as early as day 1 postnatal, the Aire-Cre transgene is already expressed in the thymus, 2-3 weeks prior to the onset of diabetes. We referred to this observation in the Discussion (Page 20, paragraph 1).

Referee #3

1. The histology of the pancreas is quite incompletely characterized ñ there is a focus on CD4 T cells only and it would be good to see staining for CD8 T cells, B cells, macrophages etc.

We examined the pancreatic sections of ID-TEC mice with antibodies targeting CD8 T-cells (anti-CD8), B cells (anti-B220) and macrophages (anti-F4/80); the results are now presented in Figure 6 (D, E and F) (Page 11). As shown, leukocytes from both adoptive (T- and B-cells) and innate compartments (macrophages) are present in the infiltrated islets.

2. The most robust test for insulin autoimmunity at present is insulin autoantibodies (IAA). It would be important to show data on IAA in the mice as well as the islet autoantibody assay that is shown.

We agreed with the reviewer that IAA would be a more stringent test to examine the presence of humoral response towards insulin in ID-TEC mice. An anti-insulin antibody-based colorometric assay was developed to examine the presence of IAA in sera of ID-TEC mice. As shown in Figure 6G (Page 11), IAAs were clearly present in ID-TEC mice.

3. The assays for T cell responses to autoantigens appear to show response to insulin but not GAD. However, a GAD peptide, GAD 206-220, defined in NOD mice was used to test for GAD responses. Given the different MHC type of the NOD and B6 mice, it is not obvious why H-2b mice would respond to this peptide. Whole GAD or GAD peptides known to be presented by H-2b would be a much better control.

Considering the reviewerís comments, we performed ELISPOT assays with the whole GAD65 protein (Abnova) and found no response greater than littermate controls. These data are now presented in Figure 6I (Page 11, paragraph 3).

4. Concerns linger about the studies involving cell transfer and thymus transplantation that have been done to confirm the autoimmune nature of the diabetes. In neither case was transfer of diabetes fully achieved although histological abnormalities were certainly present. These results raise questions about whether the presence of insulin 1 deficiency is important in developing diabetes. It would be good to see data shown on autoimmunity in mice that have thymic deletion of ins2 but ins1 is intact ñ clearly these data would have been generated in the course of the studies.

We created and analyzed Aire-cre:Ins1+/+:Ins2l/l and Aire-cre:Ins1+/-:Ins2l/l during the generation of the ID-TEC mice (Aire-cre:Ins1+/-:Ins2l/l). Neither lines developed diabetes, nor presented any islet autoimmunity as revealed by immunohistochemistry. Representative immunohistochemical analyses of pancreata from these mice are now shown in Supplemental figure S4. Clearly, thymic expression of Ins1 in Aire-cre:Ins1+/+:Ins2l/l is protective. We clarified this point in the Discussion (Page 16, paragraph 1).

5. The T cell responses to insulin could be further developed to examine the epitopes of insulin that are responsible. This could be done by replacing intact insulin with panels of insulin peptides in the interferon-gamma ELISA test shown in Fig 6E.

We examined a number of insulin peptides and found insulin B-chain peptide B9-23 as a strong stimulant for IFN- secretion in ELISPOT assays of ID-TEC splenocytes. Reactivity to B9-23 might account for the majority of IFN- secreting spots using whole human insulin as stimulant. It is of interest that B9-23 was previously found as an immunodominant peptide in H2g7 NOD mice, but not in C57BL/6 mice, which carry the same H2b haplotype as the ID-TEC mice. Recently, we
successfully isolated B9-23 specific T-cell lines from ID-TEC mice and are characterizing them.

2nd Editorial Decision

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original three referees to comment on the revised version and I have now received their reports. All three referees appreciate the carried out revisions and support publication here. Referee #1 has no further comments to the author, however referee #3 has a minor issue that I would like to ask you to respond to before final acceptance here. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

I have no further concerns.

Referee #3 (Remarks to the Author):

My main residual concern is Figure 6 of the revised manuscript. This shows islets with very few insulin-or glucagon-positive cells. There are CD4+ cells, a few CD8+ cells, a few B cells and a few macrophages. It is difficult to be sure but it seems there would be many cells in the section that do not stain for any of these. Can the authors a. comment on whether this is the case and/or b show in supplementary figures other representative sections that indicate whether the majority of the cells in the islets are accounted for by endocrine or immune stains? It would perhaps be good to show an islet with more insulin staining. Also it would be good to show serial sections of the same islet to get a better feel for the CD4 and CD8+ T cells in a single islet.

2nd Revision - authors’ response

In response to Referee #3’s request to comment on cells that are neither stained by anti-insulin antibody nor by antibodies specific for immune cells in Figure 6, our answers are as follows:

The pancreata shown in Figure 6 were harvested from 3-week old ID-TEC pups, which had already become clinically diabetic. We attributed the appearance of non-staining cells to damaged beta-cells that have lost their capacity to produce insulin. A similar situation was observed in damaged islets in new onset diabetic NOD mice. In contrast, in prediabetic ID-TEC pups, islet infiltration of various severities was observed, where most cells in islet regions were either stained with anti-insulin antibody or with anti-CD3 antibody. We present an example from our already existing data in supplementary Figure S5. We have not included these images in previous versions of our paper since we considered the quality of these images to be suboptimal for The EMBO Journal.
Specifically, we made the following changes in the manuscript:

1) A supplementary figure was added as the new S5, whereas the old S5 was renamed as S6.
2) The following sentence was added to introduce Figure S5 in the Results section: "Moreover islet infiltration of different severities was observed in pancreata of pre-diabetic 14-day old ID-TEC pups (supplementary Figure S5)" (Page 11, paragraph 1).
3) Page 15, paragraph 1. Supplementary Figure S5 was changed to S6.