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Award Number: W81XWH-09-1-0742

TITLE: Safe Gene Therapy for Type 1 Diabetes

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REPORT DATE: October 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2012	Final	28 September 2009 - 27 September 2012

4. TITLE AND SUBTITLE
Safe Gene Therapy for Type 1 Diabetes

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0742

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Pittsburgh
Pittsburgh, PA 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In light of accumulating evidence that the endocrine pancreas has regenerative properties, that hematopoietic chimerism can abrogate destruction of beta cells in autoimmune diabetes, and that in this way physiologically-sufficient endogenous insulin production can be restored in clinically-diabetic NOD mice, recapitulating what has also been sporadically seen in humans, we originally proposed to test reliable and clinically translatable alternatives able to re-establish euglycemia in diabetic patients. Instead of relying on the risky allogeneic BM transplantation to obliterate the autoimmune process that causes type 1 diabetes, we originally proposed to reconstitute, by gene supplantation, susceptible (non-Asp57+) NOD mice with their own BM genetically engineered ex vivo to also express a resistance (Asp57+) MHC class II molecule. The thymus of the reconstituted mice -- carrying BM-derived cells that co-expressed both their own diabetogenic (non-Asp57) and the transfected Asp57 beta chain -- repopulated by the engineered BM cells, can restore an efficient negative selection and consequently the ability to delete T cells potentially auto-reactive to pancreatic beta cells. These diabetics will then be disease-free.

15. SUBJECT TERMS
Type 1 diabetes; autoimmunity; bone marrow; stem cells; histocompatibility

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	87	19b. TELEPHONE NUMBER *(include area code)*

University of Pittsburgh
W81XWH-09-1-0742
Final Report (09/28/2009 – 09/27/2012)
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Our first quarterly scientific progress report for the initial year of our project (09/28/09 – 12/27/10) described the following:

Background and Significance

Low levels of peripheral tissue-specific autoantigen (PTA) expression in immune cells have been implicated as one of the many means the immune system utilizes to ensure self-recognition and maintenance of tolerance. In central lymphoid organ, the thymus, PTA expressions were found to be restricted to epithelial cells within the medulla region; and at least some of them were regulated by the autoimmune regulator (*Aire*) gene. Animals with the *Aire* gene disrupted exhibit immune cell infiltration, as well as humoral response, to multiple peripheral solid organs, including the liver, the pancreas, and the stomach. It is conceivable that loss of PTA expression/presentation within the medulla could perturb the negative selection process of PTA-specific autoreactive T cells and crumple the central tolerant mechanism. Indeed, we have demonstrated conclusively that insulin-expression in medulllary thymic epithelial cells (mTECs) is essential to mediate islet beta cell-specific immune tolerance. ID-TEC mice with insulin deletion specifically in mTECs develop autoimmune diabetes spontaneously around three weeks after birth, due to T-cell mediated beta cell specific destruction. Intriguingly, when effector T cells were adoptively transferred to immune compromised *Rag1* knockout mice, only mild hyperglycemia were observed, even though islet infiltrations of immune cells from mild to severe levels were readily detected. Similar islet autoimmunity was observed in our thymus-transplantation model, in which ID-TEC thymi were harvested and transplanted under the kidney capsule of athymic nude mice. These data implicate the existence of protective peripheral mechanism that could effectively negate the actions of peripheral PTA-specific autoreactive T cells escaped thymic negative selection.

Like the thymus, PTA expressions were also found in the stroma of peripheral lymphoid organs, including the spleen and lymph nodes. Both bone marrow-derived antigen presenting cells and epithelial cells have been implicated in peripheral tolerance induction, though direct functional evidence in naive animals is still missing. As peripheral cells (such as bone marrow cells and epithelial cells) are more accessible compared to the thymus, understanding the roles of PTA expression in peripheral lymphoid in tolerance induction would have foremost impact on developing therapeutic regimen to cure autoimmune diseases, including type 1 diabetes. With this goal in mind, we set to develop animal models to investigate the roles of insulin expression in bone marrow derived antigen presenting cells in tolerant induction towards islet beta-cells. Two animal models are being generated to address these questions: the ID-DC line in which the mouse insulin 2 gene was specifically targeted and deleted in antigen presenting dendritic cells (DCs), and the ID-BMC line, in which insulin was deleted in all bone marrow derived antigen presenting cells, including macrophages, B-cells as well as DCs. Results from these models will help us to unravel the importance of specific antigen-presenting cell types in regulating insulin autoimmunity.

Experiments in Progress

1) Progress of animal breeding to establish the ID-BMC line.

To establish the ID-BMC line, a Vav-Cre transgenic line was generated in which the promoter/enhancer elements of the mouse *vav1* gene was used to drive Cre-recombinase expression in bone marrow progenitor cells. The Vav-Cre line was first crossed to *Ins1* knockout line, and then bred to Ins2-floxed: Ins1-null mice previously generated in the lab. Of note, the Vav-Cre transgenic line can only be maintained as hemizygous as pups homozygous for the transgene perish at embryonal stage. Through multiple rounds of breeding and genetic selection, we were able to setup breeding pairs with Vav-Cre:Ins2-floxed:Ins1-null/wt (male) and homozygous Ins2-floxed: Ins1-null (female) genotypes. As predicted from Mendel's law, our current chance to get ID-BMC pups (with Vav-Cre:Ins2-floxed: Ins1-null genotype) from these breeding pairs is about 25%. At present, we were able to obtain successfully a small number of ID-BMC founders, which will be used to replace the above Vav-Cre carriers in the mating setups, to increase our chances to obtain ID-BMC pups from 25% to 50%.

2) Progress of animal breeding to establish the ID-DC line.

To establish the ID-DC line, a DC-specific CD11c-Cre transgenic line was obtained from the Jackson Laboratory and was first crossed to *Ins1* knockout line, with an approach similar to that of obtaining the ID-BMC line. At present, we were also able to obtain a small number of ID-DC pups, which will be used as founders to get more ID-DC pups for the study.

3) Initial Characterization of Vav-Cre:iYFP mice.

To validate that the Vav-Cre transgene can efficiently target the loxP-tagged Ins2 gene in bone marrow-derived cells, we crossed the Vav-Cre line to the inducible Rosa-iYFP reporter line. In Rosa-iYFP mice, transcription of the yellow fluorescent protein (YFP) was blocked by a loxp-tagged neo cassette inserted between the universal Rosa26 promoter and the YFP coding sequence. Cre-mediated depletion of the floxed neo cassette will bring the YFP sequence physically adjoining the Rosa26 promoter and turn on YFP expression. Thus, the presence of YFP in special cells can reflect faithfully the expression pattern of the tissue-specific Cre transgene.

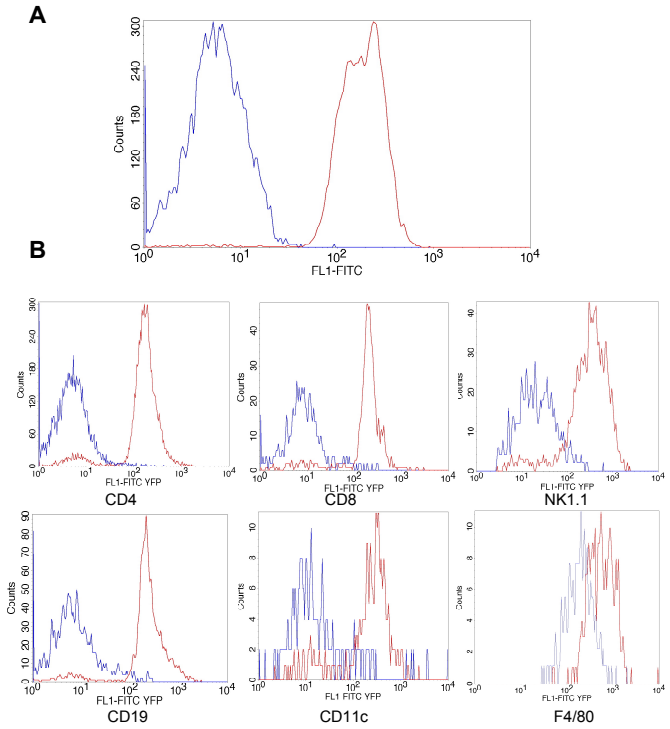

Figure 1. Characterization of Vav-Cre: iYFP mice. A. Peripheral blood samples were harvested and treated with red cell-lysis buffer and stained with anti-CD45 antibody. CD45+ cells were analyzed for YFP expression. Red line: Vav-Cre:iYFP; Blue line: Rosa-iYFP control. B. Splenocytes harvested from Vav-Cre:iYFP mice were stained with CD4, CD8, NK1.1, CD19 CD11c and F4/80 antibodies together with CD45 antibody analyzed by FACS for YFP expression (x-axis). Cells were gated for double positive of CD45+ and the antibody shown underneath each panel.

YFP expression in peripheral blood mononuclear cells (PBMCs) was first evaluated in Vav-Cre:iYFP mice. As shown in Figure 1A, almost all the PBMCs are YFP-positive, indicating high efficiency of the Vav-Cre transgene in hematopoietic lineages (**Figure 1A**), consistent with our previous results using Rosa-iLacZ as

reporter line. To further characterize the Vav-Cre expression in specific hematopoietic cell types, secondary lymphoid organs (including the spleen and the lymph nodes) were harvested and YFP expressions in specific cell types were characterized with FACS analysis. As shown in **Figure 1B**, YFP molecules are present in more than 95% of CD4+T cells, CD8+ T cells, B cells and NK cells. Of note, professional antigen presenting cells (APCs), including CD11c+ dendritic cells (DCs) and F4/80+ macrophages, also exhibit high percentage of YFP-positivity (~93% and 100%, respectively). Similar results were obtained from cells harvested from lymph nodes.

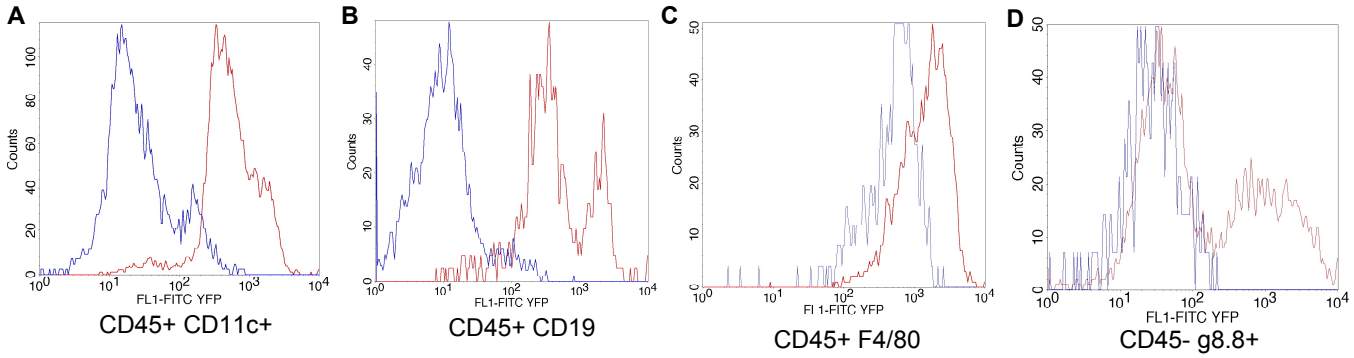

Figure 2. Characterization of YFP expression in the thymi of Vav-Cre:iYFP mice. Thymi harvested from Vav-Cre:iYFP mice were digested with colleagenase to single cell suspension. After CD90 magnetic bead-depletion of thymocytes, cells were further separated into CD45+ bone marrow derived and CD45- epithelial populations. CD45+ cells were stained CD11c, CD 19 and F4/80 antibodies, respectively; whereas CD45- cells were stained with g8.8 and Ly51. YFP expression in specific cell types (gated as labeled under each panel) was evaluated by FACS. Red line: Vav-Cre:iYFP; Blue line: Rosa-iYFP control.

YFP expression pattern in thymi harvested from the Vav-Cre:iYFP mice was also examined. Consistent with results from the periphery, Vav-Cre transgene can efficiently turn on YFP expression in thymic APCs (including DCs, macrophages and B cells). We also examined CD45- thymic stromal cells (**Figure 2A-2C**). About 65% of the CD45-
g8.8+ thymic epithelial cells are negative for YFP (**Figure 2D**). Further characterization of these cells showed that YFP-negative cells belonged to the Ly51 low compartment (data not shown), indicating that Vav-Cre is not expressed in thymic medullary epithelial cells. All in all, these data indicate that the Vav-Cre transgene can efficiently delete the floxed Ins2 gene in APCs derived from hematopoietic lineage in both central and peripheral immune organs.

3) Results from preliminary characterization of ID-BMC mice.

With the small number of ID-BMC mice obtained, we were able to do a number of experiments to characterize them preliminarily. First, we directly examine the efficiency of the Vav-Cre transgene mediating deletion of the floxed Ins2 gene. As shown in Figure 3A, Southern blot analysis of genomic DNA harvested from bone marrow progenitor cells of ID-BMC mice showed almost 100% deletion of the floxed Ins2 allele. However, RT-PCR analysis of thymi harvested from 6-week old ID-BMC mice showed that thymic insulin expression is largely intact in ID-BMC mice (**Figure 3B**), suggesting that within the thymus, the majority of the Ins2 transcripts observed are derived from mTECs. These animals are normoglycemic at 6-weeks, and we are closely monitoring their blood glucose levels on weekly basis.

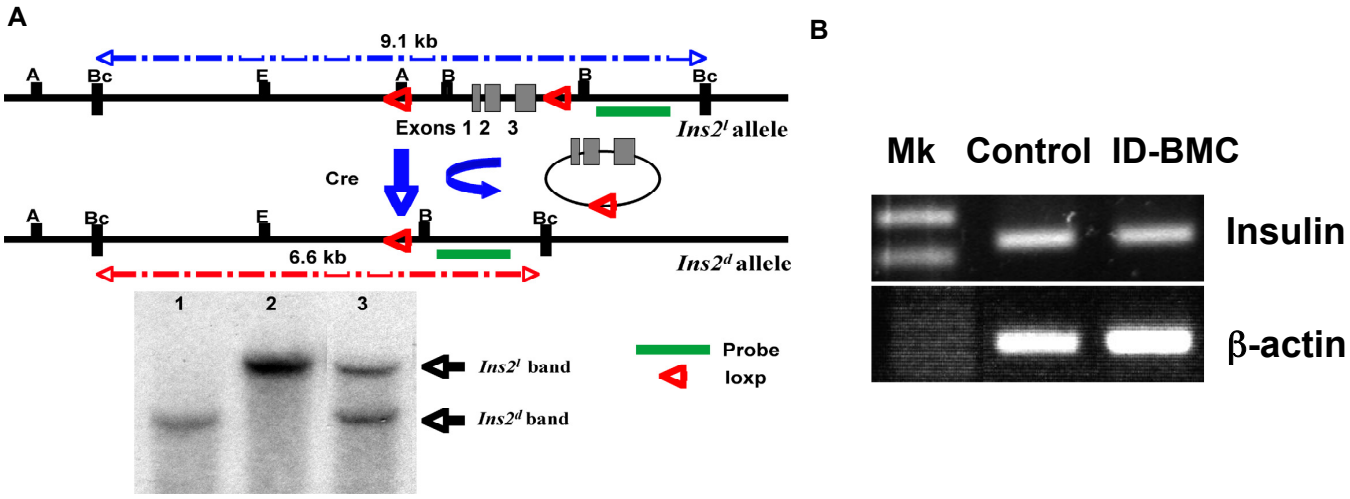

Figure 3. Characterization of ID-BMC mice: A preliminary study. A. Southern blot analysis of genomic DNA harvested from bone marrow progenitor cells of ID-BMC mice. *Upper panel:* Schematic drawing of the Ins2 floxed allele and the Ins2 deletion allele. Restriction endonucleases: A, AseI; Bc, BclI; B, BamHI; E, EcoRI. Cre mediated action will eliminate the Ins2 DNA fragment between the two flanking loxp sites, resulting a shorter fragment compared to the floxed allele (6.6kb vs 9.1kb) when genomic DNA samples were digested with BclI. Red triangle, loxp site; Green line, probe for Southern analysis. Lower panel: Southern blot showing the nearly total deletion of the Ins2-floxed allele in ID-BMC bone marrow cells (lane 1, upper band). Lane 2, DNA from bone marrow of homozygous floxed mice. Lane 3, DNAA harvested from bone marrow of heterozygous mice with one Ins2 deletion allele and one floxed allele. B. RT-PCR analysis of thymic insulin expression in ID-BMC mice. Mk. Molecular marker. Comparable levels of insulin expression were found in thymi of control (Ins2-floxed, Ins1-null) littermates and ID-BMC mice.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

1) We have proved that the Vav-Cre transgene can efficiently target Cre-mediated deletion in all hematopoietic compartment, especially in antigen-presenting cells within both the primary and secondary lymphoid organs, but not in medullary epithelial cells. Our breeding scheme to generate both ID-BMC and ID-DC lines are successful and we have already obtained a small number of founder animals. These founder animals will enable us to get more ID-BMC and ID-DC mice for experimental purposes and speed up the study.

2) As predicted, the Vav-Cre transgene can efficiently mediate recombinant events in all cell types of the hematopoietic lineages. Especially, it enables high efficient deletion of insulin in antigen presenting cells of bone marrow origin in both central and periphery organs. Such feature will allow us to investigate the immunologic importance of insulin ectopic expression bone marrow derived APCs in mediating self-tolerance.

3) Our preliminary results from a small number of ID-BMC animals suggest that bone marrow-derived APCs are not the major source of thymic insulin transcripts. No major change of levels of insulin expression is detected, even though near total deletion of the floxed Ins2 alleles was achieved in all bone marrow derived cells. We are in the process of further evaluate insulin autoimmunity in these animals.

Our second quarterly scientific progress report for the initial year of our project (12/28/09 – 03/27/10) described the following:

Background and Significance

Peripheral tissue-specific antigen (PTA) expression in immune cells of the central or peripheral lymphoid organs has been implicated in establishing and maintaining immune tolerance towards self. We and other have demonstrated conclusively that abrogation of a single PTA in medullary thymic epithelial cells (mTECs) is sufficient to trigger tissue-specific autoimmunity. Recently, attentions have also been drawn to the non-hemaetopoietic stromal compartments in peripheral lymphoid organs, as above-noise levels of PTA transcripts were detected. Stromal cell types expressing different surface markers or transcription factors were shown to possess the properties of PTA-production, and their roles in modulating peripheral tolerance towards different tissues and organs were heavily debated. Anderson and colleagues isolated EpCAM+, MHC II+, Aire+ eTACs (extrathymic Aire-expressing cells) and showed that a diverse array of PTAs were expressed in these cells and they were capable to interact with and delete autoreactive T cells. In contrast, Engelhard's group from University of Virginia identified a population of gp38+MHCII+ lymph node stromal cells (LNSC) that can directly express and present multiple PTAs. Interactions between autoreactive cytotoxic CD8+ T cells specific to a melanoma-specific antigen (MSA) with MSA-expressing LNSCs induce programmed cell death of the T cells. Intriguingly, Turley's group from Harvard University reported recently that more than one stromal cell types in the lymph nodes possessed the PTA-expressing, tolerogenic induction properties; and that levels and diversity of PTA expression in the stroma are responding to environmental cues, such as virus infection and inflammation. Nevertheless, these new findings suggest the existence of another checkpoint mechanism in the immune system to maintain self-tolerance.

In this quarter, we continued to characterize the ID-BMC and ID-DC mouse models as mentioned previously. In addition, we also took advantage our Aire-Cre transgenic line to examine the presence of Aire-expressing cells in the periphery. Specifically, we crossed Aire-Cre transgenic mouse to RosaYFP reporter mouse to generate the Aire-Cre:RosaYFP. As mentioned previously, YFP expression was blocked by a stopper sequence that flanks the constitutively active Rosa26 promoter and the YFP coding sequence in RosaYFP mouse. Cre-recombinase molecules translated from the Aire-cre transgene could efficiently delete the stopper sequence, and acted as a molecular switch to permanently turn on YFP expression. Thus, YFP can be used as a molecular tracer to truthfully record the developmental history of the Aire-expressing cells. Here, we reported our findings within the 1st quarter.

Experiments in Progress

1) Characterization of ID-BMC mice.

We have shown from a small number of ID-BMC animals that bone marrow-derived antigen-presenting cells are not the major source of thymic insulin transcripts. No major change of levels of insulin expression is detected, even though near total deletion of the floxed Ins2 alleles was achieved in all bone marrow derived cells. ID-BMC mice remain euglycemic for more than 16 weeks; and we did not observe any incidence of hyperglycemia, in striking contrast to the 100% penetration of spontaneous diabetes observed in our mTEC-specific insulin-deletion ID-TEC colony three weeks postnatal. Furthermore, we did not observe any CD4+ T-cell infiltration or insulitis in the pancreata of ID-BMC mice, indicating the absence of any islet autoimmunity (Figure 1). We are currently developing in vitro assays to directly examine the presence/absence of anti-insulin specific T cells in ID-BMC mice.

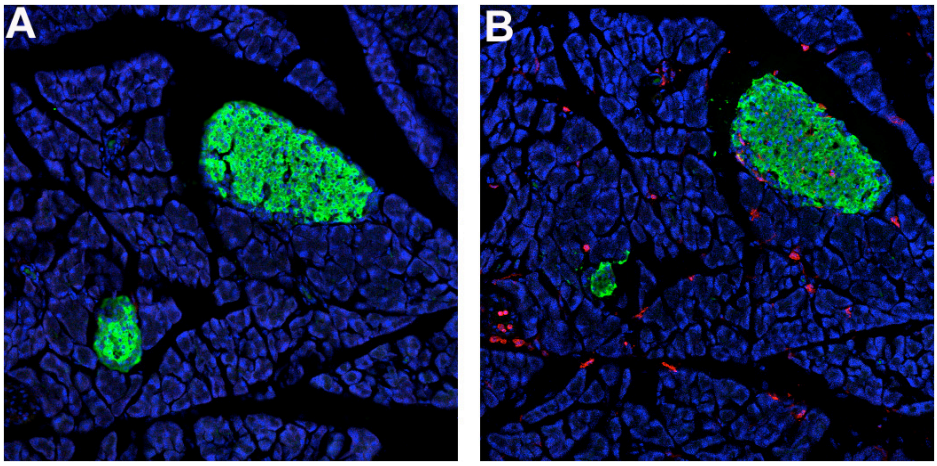

Figure 1. Immunohistochemical analysis of pancreata harvested from ID-BMC mice. Pancreata harvested from 12-week old ID-BMC mice were fixed with 4% paraformaldehyde. 5um cyrosections were stained with anti-insulin (green), anti-CD4 (red, **A**), and anti-CD45 (red, **B**) antibodies. Although CD45+ residue immune cells are readily detectable, no CD4+ T-cells was observed infiltrating the pancreas.

2) Characterization of the ID-DC lines.

With the small number of founder animals, we were able to expand a limited number of ID-DC mice. Similar to the ID-BMC line, all the ID-DC mice remain euglycemic for more than 8-weeks. We are currently expanding the colony to further characterize the animals both *in vitro* and *in vivo*.

3) Characterization of the Aire-Cre:YFP mice.

As the first step, we harvested solid organs from the Aire-Cre:YFP mice and examined the YFP expression by histology. Consistent with our previous results, no co-staining of YFP signal and insulin was observed, indicating that Aire-Cre was not expressed in insulin-secreting pancreatic beta-cells (Figure 2). Interestingly, we did observe YFP positive cells in the spleen (Figure 2, spleen section on the top), consistent with previous findings of the presence of PTA-expressing cells. Interestingly, YFP positive cells were not detected in the white pulp region, where active immune humoral and cellular responses occur, but were largely present in the marginal zone bordering the white pulp. Such geometrical location are consistent with a tolerogenic role of Aire-expressing cells where naive T cells were first interrogated by self-antigens before they migrate into white pulp to elicit active immune response. Sparse YFP positive cells were also observed in a number of solid organs and tissues, including the lung, kidney, stomach and the intestines, but not observed in brain or heart (Figure 2). Morphological examination suggests that most of them are epithelial cells, and there was no clonal clusters, indicating that the YFP positive cells are not necessarily from the same ancestral lineage. Indeed, it was recently shown that Aire-mediated expression of PTA was also observed in the epithelium of the thyroid, suggesting that random PTA-expression is a general property of certain epithelial cells. However, its immunologic significance in regulating self-tolerance remains to be examined.

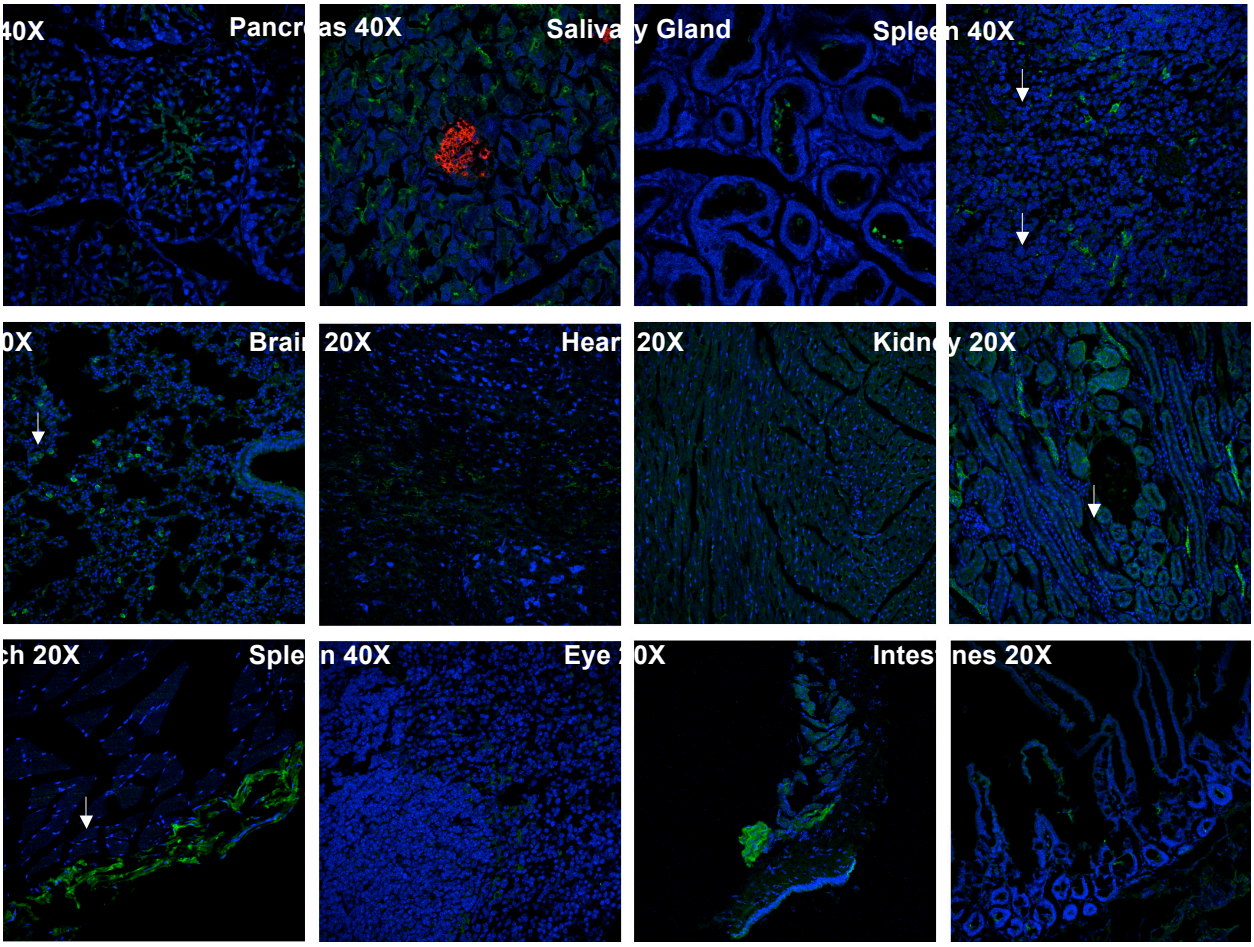

Figure 2. Immunohistochemical analysis of patterns of Aire-Cre expression. Solid organs harvested from Aire-Cre:RosaYFP mice were fixed in 4% paraformaldehyde and cyrosections of 5um thick were stained with rabbit anti-GFP polyclonal antibodies (green, all the slides), as well as anti-insulin antibody (the pancreas section). White arrows indicate the YFP-positive cells in the spleen, lung, stomach and kidney.

Another interesting findings in our histological characterization of the Aire-expression pattern is the presence of YFP signal in mature spermatozoa, but not in immature spermatids. The presence of YFP signal in the male reproduction system raised the question: how Aire-Cre transgene retain its specificity of mTEC-specific Cre expression if the transgene is active in the sperm. One potential explanation is that the tight compaction of chromosomal DNA in mature sperm is resistant to Cre-mediated deletion of the loxp-flanking DNA element. Furthermore, the small amount of Cre-recombinase protein introduced into the fertilized egg is insufficient to mediate efficient homologous recombination.

4**) YFP expression in Aire-Cre:RosaYFP thymus.**

We also examine patterns of YFP expression in Aire-Cre:RosaYFP stroma. CD45-EpCAM+MHCII+ thymic epithelial cells (TECs) were harvested and examined by FACS analysis. As shown in Figure 3, about 68% of the TEC cells are YFP-positive, consistent with our observation that 60-70% of TECs isolated in our protocol are mTECs. These results suggest that our Aire-Cre:RosaYFP model can efficiently label all the mTECs in the thymus. Interestingly, we also observed ~8% of thymic CD11c+ dendritic cells (DCs) are YFP positive. Indeed, low levels of Aire transcripts were previously detected in thymic DCs. Alternatively, these YFP signals were derived from engulfing of mTEC components by thymic DCs for cross-presentation. Nevertheless, our Aire-Cre:RosaYFP can faithfully reproduce the pattern of Aire expression pattern in the thymus, and can efficiently label all the mTECs.

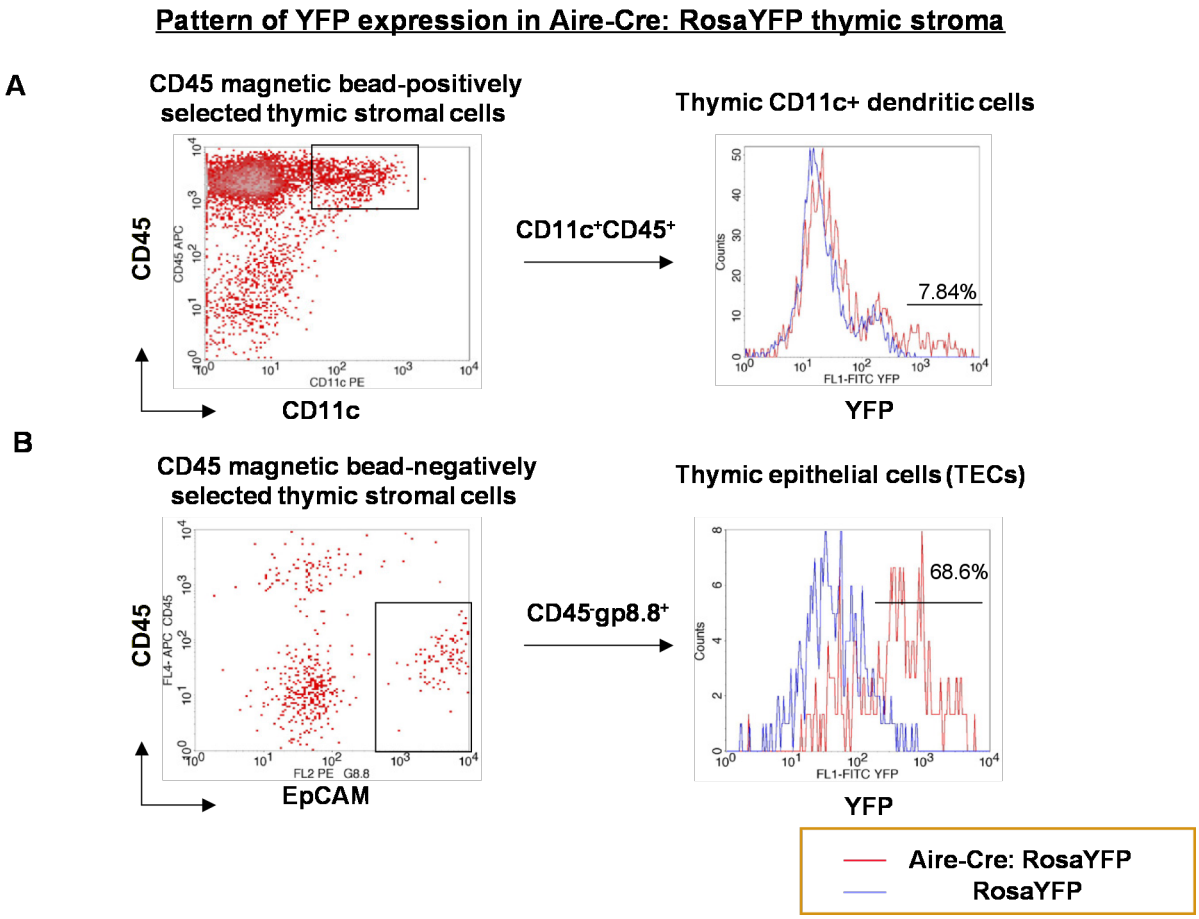

Figure 3. Aire-expressing cells in thymic stroma. Aire-Cre:RosaYFP thymi were harvested and separated into single cells via collagenase digestion, followed by CD90 magnetic bead-depletion of thymocytes. The negatively selected cells were further separated by CD45 antibody conjugated magnetic beads into the CD45+ bone marrow-derived cells (A) and the CD45- epithelial cell population (B). As shown in Panel B, about 60-70% of TECs are YFP positive, which is consistent with the 2:1 mTEC to cTEC ratio, which we normally observed during TEC isolation (with Ly51 and UEA-1). We also routinely found a small percentage of thymic dendritic cells (CD4-CD45+CD11c+) that are YFP positive (~7-8%).

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12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

1) We expanded our studies on ID-BMC mice during this quarter, and did not observe any pathological signs of diabetes in these animals. Furthermore, no CD4+ T cell infiltration was observed in ID-BMC pancreas, suggesting that deletion of insulin in bone marrow derived antigen presenting cells are not essential to establish immune tolerance towards islet beta cells.

2) We have also established an Aire-Cre:RosaYFP animal model, which can faithfully reproduce the expression patterns of the Aire gene in the thymus. Our immunohistochemical characterization of the YFP expression patterns in peripheral tissues and solid organs revealed the interesting finding that Aire is also sparsely expressed in epithelial cells of a variety of tissues and organs.

3) <u>In the next quarter</u> we will use now the Aire-Cre:RosaYFP animals as a useful tool to isolate and characterize PTA-expressing cells in both the central and peripheral lymphoid organs, which will significantly facilitate our understanding of the underlying mechanisms of establishing and maintaining immune tolerance towards self.

Our third quarterly scientific progress report for the initial year of our project (03/28/10 – 06/27/10) was waived since a detailed report was presented at the Diabetes and Chronic Disease Product Line Review on June 15, 2010. We are including a summary of the slides presented there.

Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

15 June 2010

1

Safe Gene Therapy for Type 1 Diabetes

Dr. Massimo Trucco

Principal Investigator

28 Sept 09– 27 Oct 11

$3,604,000.00

CSI

Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

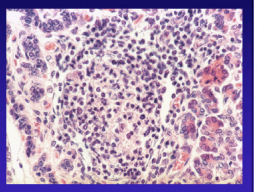
15 June 2010

2

Military relevant issue to be solved

Insulin -Dependent Diabetes Mellitus
(Type 1 Diabetes)

T1D is a systemic disease, characterized by hyperglycemia, hyperlipidemia and hyperamino-acidemia.
It is caused by a decrease in the secretion of insulin, due to the destruction of the β cells of the pancreas.
It is frequently associated with specific lesions of the microcirculation, neuropathic disorders and a predisposition to atherosclerosis.



Human islet of Langerhans with insulitis
Trucco et al. CRC Reviews 9:201, 1989

Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

15 June 2010

3

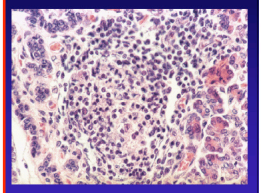
Military relevant issue to be solved

Insulin -Dependent Diabetes Mellitus
(Type 1 Diabetes)

Sixteen million people in the US have diabetes with 800,000 new cases diagnosed each year.

Diabetic complications threatening vision, kidney, and nerve function affect nearly all diabetic patients.

The military population closely reflects the situation of the country.



Human islet of Langerhans with insulitis
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Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

15 June 2010

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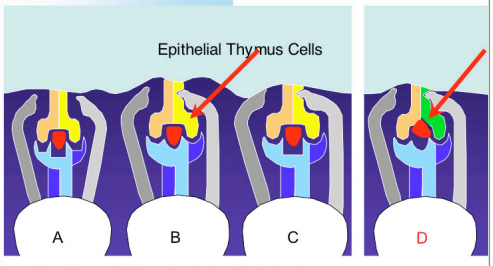
Solution



Trucco & Giannoukakis *Gene Therapy* 12:553, 2005

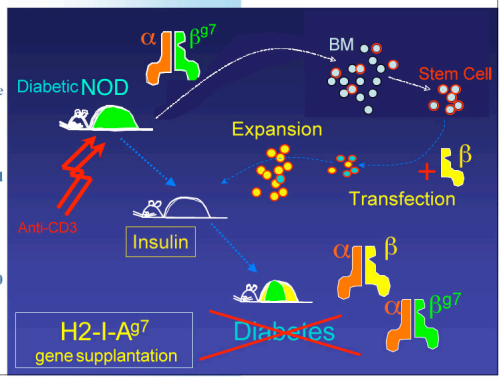
Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

15 June 2010

5

Project Description



Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

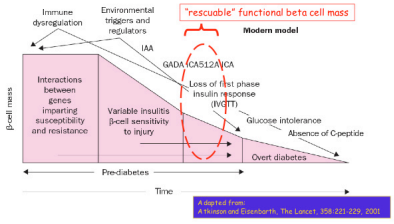
15 June 2010

6

Project Description

Working principle:

"Rescue and/or regeneration of functional residual beta cell mass can reverse type 1 diabetes"



A digest from: A Hinson and Eisenbarth, The Lancet, 358:221-229, 2001

•A number of studies in the NOD mouse model have proven that residual beta cell mass can be rescued by interfering with autoimmunity; prevention of further loss of functional mass facilitates resumption of euglycemia

•Rescue of residual beta cell mass may concurrently facilitate endocrine cell regeneration replenishing lost/ dysfunctional beta cell mass to variable degrees; this too can participate in "reversal" of new-onset disease

Project Description

Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

15 June 2010

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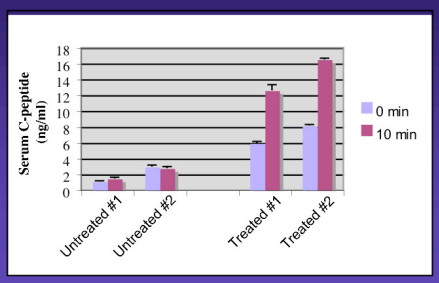
Endogenous C-Peptide in treated NOD Mice



Project Description

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Diabetes and Chronic Disease: Filling the Gaps

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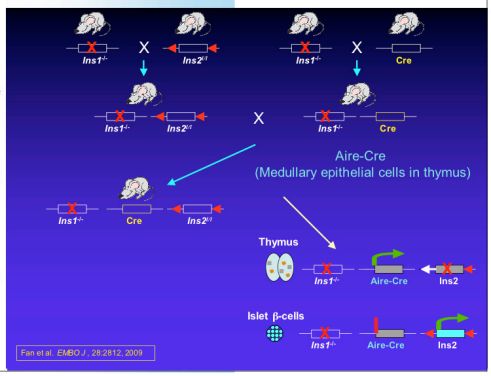

Project Description

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Diabetes and Chronic Disease: Filling the Gaps

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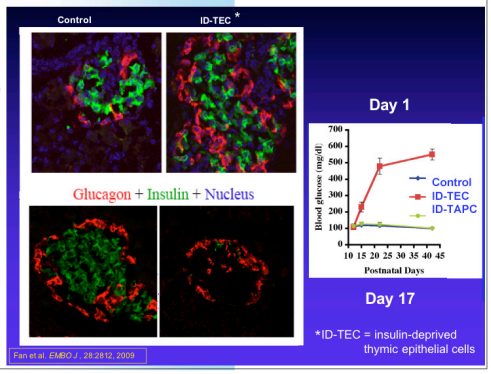

Validation Strategy

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Diabetes and Chronic Disease: Filling the Gaps

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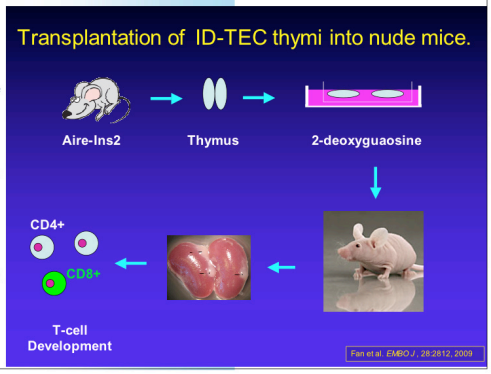

Validation Strategy

Product Line Review (PLR) Meeting

Diabetes and Chronic Disease: Filling the Gaps

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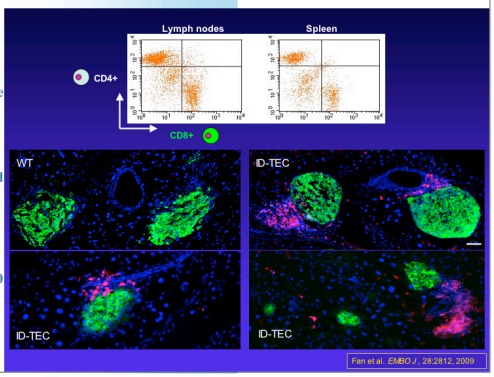

Include detailed timeline/Gantt chart

Research/Development Timeline

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Milestone #1: To demonstrate whether BM derived cells carrying the H2 molecule able to confer resistance to type 1 diabetes (I.E., our "treatment") need to also express self-antigens to properly present them and complete negative selection, or have to capture them from other cells for cross presentation. The strategy in which T1D can be reverted by substituting a "diabetes-susceptible" class II MHC beta. — May 2010

Milestone #2: To test the efficacy of the treatment in terms of its ability to allow the regenerative capabilities of the diabetic pancreas to recover sufficient insulin production for controlling the animal glycemia in new-onset diabetic NOD mice, while characterizing the immune scenario that favors this regeneration (e.g., regulatory T-cell expansion). — September 2010

Milestone #3: To ascertain the efficacy of engraftment and repopulation capabilities of the engineered hematopoietic precursor cells, following non-radiation based pre-conditioning. A combination of two antibodies (anti-c-kit and anti-CD52) will be used instead. — December 2010

Milestone #4: To substitute non-viral minicircle DNAs for retroviruses as the vectors for transfecting the protective histocompatibility H2 I-A beta chain transgene into enriched autologous BM hematopoietic cell precursors. — May 2011

Milestone #5: To re-program differentiated pancreatic exocrine cells of autoimmunity-free NOD mice into cells that "closely resemble beta cells". — September 2011

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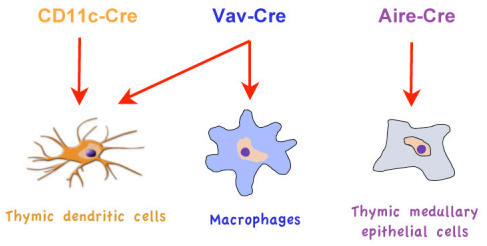
Successes to Date

To target specific types of antigen presenting cells in the thymic stroma, three transgenic animal lines were generated:



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Intellectual Property / Publications Deriving from this Project

- Confidentiality Agreements:
  - Materials Transfer Agreements were signed between the University of Pittsburgh and a number of other Institutes:
  - –National Institute of Health (Dr. Hodes).
  - –University of Calgary, Canada (Dr. Santamaria).
  - –Washington University, St. Louis (Dr. Moley)
- Patents Filed:
  - –None at present, but we are certainly aware of the possibilities when our research move along further.

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Intellectual Property / Publications Deriving from this Project

- List all Publications deriving from the project:

Fan Y, Rudert WA, Grupillo M, He J, Sisino G and Trucco M. "Thymus-specific deletion of insulin induces autoimmune diabetes". **The EMBO Journal 28: 2812-2824, 2009.**

Feature article of the issue.
Selected at NIH as Faculty of 1000 exceptional paper with F1000 factor 9.0
Selected for the 2010 Senior Vice Chancellor's Research Seminar Pitt. Univ.

Two manuscripts are currently under preparation:

Ecotopic insulin expression in bone marrow derived antigen presenting cells is not indispensable for maintaining immune tolerance towards beta cells.

Self-tolerance mediated by peripheral tissue specific antigen expression: the Aire experience.

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Project Funding

Current Budget	Expended Funds
$ 2,007,693	$ 392,384 (out of $ 1,561,796 received to date)

Other Funding if applicable

NIH, 1 U01 DK61058-01
Title: Prediction and Prevention of Type 1 Diabetes
Annual Direct Costs: 09/01/09 -- 08/31/10; $ 315,019
Entire Period: 09/01/09 - 08/31/14; $ 2,519,975

Henry Hillman Endowed Chair to M. Trucco
$ 382,360

Cochrane Webber Endowment to Yong Fan
$ 25,000

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Additional Project Information

Lab/Company/Group: University of Pittsburgh
Principal Investigator: Dr. Massimo Trucco
Government COR: Mr. Robert Read
Government Project Officer: Stacy Zimmerman
Contract Instrument: Grant
Period of Performance: 28 Sept 09 – 27 Sept 11
Contract Specialist: Ms. Nita Bourne
EDMS# : 4235
Contract #: W81XWH0910742

In the fourth quarterly scientific progress report (06/28/10 - 09/27/10) of year 01, we now report on our cumulative results.

Background and Significance

In addition to pancreatic islets, low levels of insulin expression were found in a number of tissues and organs, including but not limited to the thymus, the brain, the salivary glands, the testis and the bone marrow. Although such extrapancreatic insulin production has been implicated in regulating many biological processes, such as maintaining metabolic and neuroendocrinological homeostasis under either physiological or pathological conditions, experimental evidence supporting these claims are mostly missing. We have a long interest in understanding the roles of insulin expression in cells of immune organs, and their implication in type 1 diabetes etiology. As described in previous reports, our ID-TEC animal model, in which insulin expression in medullary epithelial cells of the thymus (mTECs) is specifically targeted and depleted, demonstrated conclusively that thymic insulin production, albeit at low levels, is essential to establish adaptive immune tolerance of islet beta cells by eliminating insulin-specific autoreactive T cells through central negative selection mechanisms. Interestingly, insulin expression in immune-relevant organs is not restricted to epithelial cells of the thymic medulla. The presence of insulin transcripts in bone marrow derived antigen presenting cells have been reported by a number of groups. Taking advantage of the Aire-Cre:Rosa-YFP reporter line developed in our laboratory, we were able to identify at least two types of cells with antigen-presenting capability (expressing MHC II molecules) in secondary lymphoid organs: the $CD45^{+}CD11c^{int}MHCII^{+}$ cells in the spleen and the $CD45^{-}MHCII^{+}$ cells in stroma of lymph nodes (as reported in our third quarterly scientific report).
As reported in the previous quarters, we had generated a number of animal models, including the insulin deletion in bone marrow derived cells line (ID-BMC) and the insulin deletion in dendritic cells line (ID-DC), to investigate the immunologic significance of insulin expression in bone marrow derived antigen presenting cells. In this quarter, we continued our characterization of the ID-BMC and ID-DC lines, and conclude that depletion of insulin expression in antigen presenting cells of bone marrow origin is not sufficient to induce anti-insulin autoimmune response. In addition, we successfully introduced the autoimmune diabetes susceptible $H2^{g7}$ MHC alleles from the NOD mice into the ID-DC lines, to evaluate the potential roles of insulin expression in DCs in preventing uncontrolled expansion of autoreactive T cells arisen from the faulty thymic negative selection. We will also present our results of initial characterization of these mice in this quarterly report.

Results of Experiments in Progress

1) Characterization of ID-BMC mice

As previously described, ID-BMC mice were born healthy with no obvious physiologic abnormalities, and did not show any sign of dysregulation of glycemic control throughout their lifespan (Figure 1, *left panel*). Compared with littermate controls, ID-BMC mice display normal response to glucose challenge, and normal insulin sensitivity, suggesting that there are no abnormalities in glucose metabolism and islet function (Figure 1, *right panel*). Consistently, normal islet structure and insulin production were observed in pancreata harvested from ID-BMC mice. We did not observe any damaged islets nor islets infiltrated with immune cells (a.k.a. insulitis), indicating the absence of anti-islet autoimmunity. Furthermore, compared with controls, no above the background levels of insulin-specific autoreactive T cells was observed in ELISPOT assays of ID-BMC splenocytes, using insulin or its peptides as stimulants. Thus, elimination of insulin expression in bone marrow derived antigen presenting cells is insufficient to break down immune tolerance towards insulin to trigger anti-islet autoimmunity.

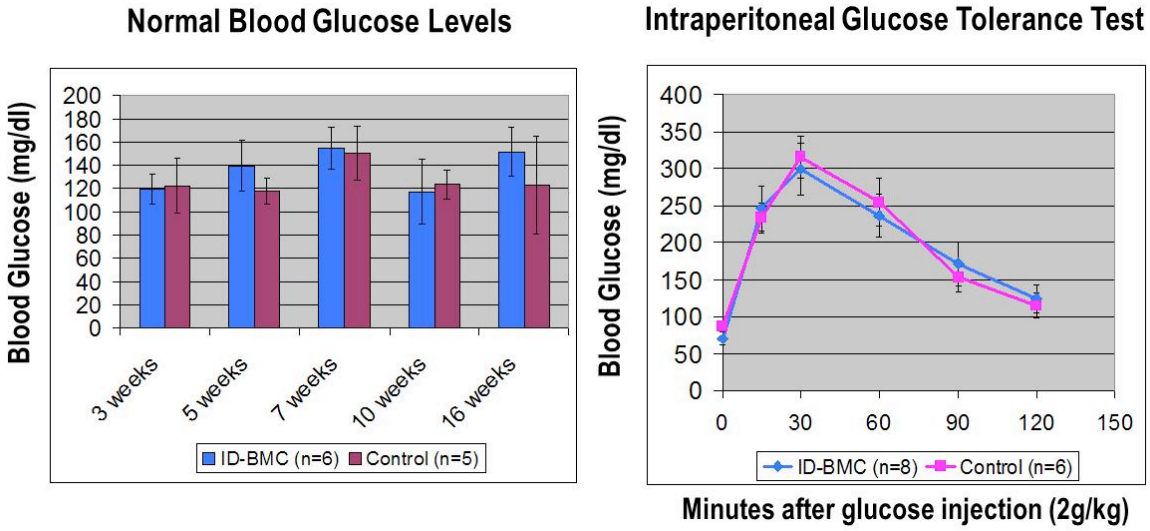


Figure 1. ID-BMC mice can maintain normal glucose homeostasis. *Left panel*, ID-BMC mice display normal levels of blood glucose levels, as compared with controls. *Right panel*, 16-week old ID-BMC mice were fasted overnight, and challenged with intraperitoneal injection of glucose (2g/kg body weight). No difference was observed between ID-BMC mice and controls, indicating that ID-BMC mice can efficiently maintain glucose homeostasis.

2) Insulin expression in plasmacytoid dendritic cells (pDCs) within the spleen.

As low levels of Aire gene expression were was found in bone marrow-derived CD11c+ dendritic cells within the thymus, we took advantage of the fact that Aire-Cre transgene can faithfully recapitulate the endogenous Aire gene expression pattern and investigated whether a similar Aire^{+} CD11c^{+} DC population were present in secondary lymphoid organs. Aire-Cre transgenic mice were crossed to reporter RosaYFP mice to generate the Aire-Cre:RosaYFP line. Indeed, about 1% of the CD45+ splenocytes, with the molecular signature of tolerogenic, plasmacytoid DCs (CD11c^{int}, MHCII^{+}), are YFP+ (Figure 2, *left panel*). Interestingly, qPCR analysis of insulin gene expression revealed that insulin transcripts are predominantly present in CD11c^{int}YFP^{+} of cells, but not in CD11c^{high}YFP^{-} DCs (Figure 2, *right panel*), indicating that, unlike thymus, insulin expressions in the spleen are largely restricted to Aire^{+}, plasmacytoid DCs of bone marrow origin.

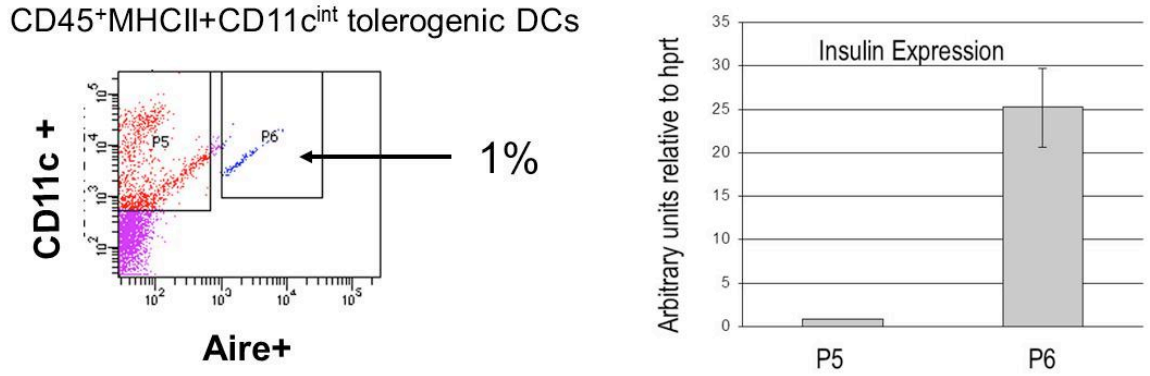


Figure 2. Insulin is expressed in Aire^{+}MHCII^{+}CD45^{+}CD11c^{int} plasmacytoid DCs in the spleen. *Left panel*, FACS analysis showed that about 1% of the CD45+ splenocytes (box marked with P6) express Aire and intermediate levels of CD11c, the molecular signature of tolerogenic plasmacytoid DCs. *Right panel*, qPCR analysis of insulin expression levels in classic CD11c^{high} DCs (P5) and plasmacytoid, Aire+ DCs (P6), showing that insulin is predominantly expressed in Aire+ pDCs in the spleen.

3) **Characterization of ID-DC and ID-DC-H2g7 mice.**

To further investigate the function of insulin-expression in Aire^{+}CD11c^{int} DCs in the spleen, we crossed transgenic mice expressing Cre-recombinase under the CD11c promoter (CD11c-Cre) to Ins2$^{l/l}$:Ins1$^{d/d}$ mice to generate ID-DC mice, as previously described. Similar to ID-BMC mice, ID-DC mice remain euglycemic throughout life, with no signs of compromised glucose metabolism or islet autoimmunity (data not shown). As both ID-BMC and ID-ADC are under C57BL/6 background with autoimmune diabetes resistant MHC (H2b haplotype), which can efficiently present autoantigens within the thymic medulla to mediate central negative selection, autoreactive T cells with high-affinity to islet autoantigens were proficiently eliminated within the thymus. Thus, under conditions of effective thymic negative selection, deletion of insulin expression in tolerogenic DCs in the periphery will not induce anti-islet autoimmunity.

To examine the immune tolerogenic roles of insulin expression in Aire^{+}CD11c^{int} DCs under faulty central negative selection conditions, we introduced the autoimmune diabetes-susceptible H2^{g7} MHC alleles of the non-obese diabetic (NOD) mice to ID-DC mice through multiple rounds of breeding with B6-H2^{g7} congenic mice to generate ID-DC-H2g7 mice. ID-DC-H2g7 mice displayed similar levels of blood glucose as non-Cre littermate controls (Figure 3, *left panel*). As well, no difference of glucose metabolism was observed when challenged with intraperitoneal glucose injection (Figure 3, *right panel*), suggesting normal islet function.

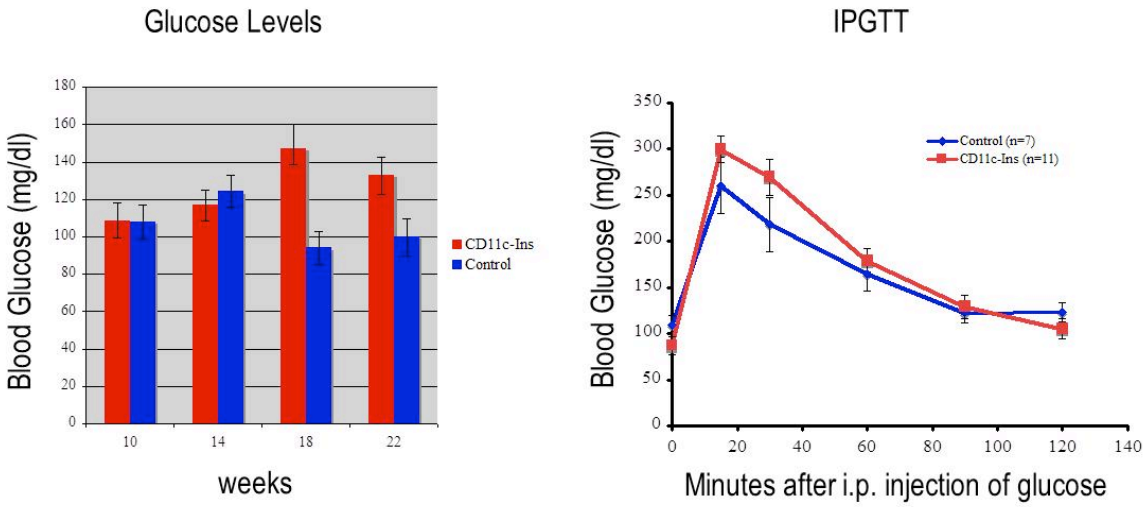


Figure 3. Normal glucose metabolism in ID-DC-H2g7 mice. Left panel, ID-DC-H2g7 animals remain euglycemia. Right panel, Intraperitoneal glucose tolerance test (IPGTT). ID-DC-H2g7 mice display normal tolerance to glucose challenge, as compared with controls.

Plans or milestones for the next quarter:

1) Our results from characterization of ID-BMC mice clearly demonstrated that depletion of insulin in bone marrow derived antigen presenting cells is not sufficient to breach either central or peripheral immune tolerogenic mechanisms in animals with autoimmune diabetes-resistant MHC. Our data also emphasize the dominant roles of central mechanism in establishing adaptive immune tolerance towards pancreatic beta cells. Thus, our focus in the future should be shifted to the roles of insulin expression in secondary lymphoid organs in preventing clonal expansion of insulin-specific autoreactive T cells which escaped negative selection.

2) We were able to identify a population of plasmacytoid DCs in the spleen which express both Aire and insulin. Further characterization of this population will allow us to explore the possibility of using these cells to strengthen peripheral tolerance to insulin, which might lead to development of individualized therapeutic vaccines to prevent insulin autoimmunity.

3) Although our initial characterization of the ID-DC-H2g7 mice did not display any abnormalities of islet function at physiological levels, we will investigate whether these animals display any anti-islet autoimmunity in the next quarter.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have successfully generated and characterized the ID-BMC animal model in which insulin expression is abrogated only in bone marrow derived cells. We further demonstrated that the absence of insulin in bone marrow cells is not sufficient to induce autoimmune diabetes, thus, emphasize the dominant roles of central mechanism in T1D etiolgoy.

2. We have identified two insulin-expressing stromal cell types in secondary lymphoid organs, and we will explore their therapeutic potency to strengthen peripheral tolerogenic mechanism to counteract preexisting faulty thymic negative selection conditions.

3. We have successfully generated ID-DC and ID-DC-H2g7 animals, in which insulin expression is specifically knocked out in both classic and tolerogenic dendritic cells. These animals will allow us to dissect further the interactions between central and peripheral immune mechanisms in establishing/regaining insulin tolerance.

REPORTABLE OUTCOMES:

Manuscripts (5 publications)

1. Lu C, Kumar PA, **Fan Y**, Sperling MA, Menon R (2010) A novel effect of GH on macrophage modulates macrophage-dependent adipocyte differentiation. *Endocrinology* 151 (5): 2189-2199.

2. Perdomo G, Kim DH, Zhang T, Qu S, Thomas EA, Toledo FG, Slusher S, **Fan Y**, Kelley DE, Dong H (2010). A role of apolipoprotein D in triglyceride metabolism. *Journal of Lipid Research* 51: 1298-1311.

3. Mavalli M, DiGirolamo D, **Fan Y**, Riddle R, Campbell K, Sperling M, Frank S, Bamman M, Clemens T (2010). Distinct growth hormone receptor signaling modes control skeletal muscle development and insulin sensitivity. Manuscript accepted for publication in the *Journal of Clinical Investigation*. *J Clin Invest.* 2010; doi:10.1172/JCI42447.

4. Trucco M (2010). Beta-cell regeneration: from science fiction to challenging reality. *Pediatr Diabetes* 11(5):292-5.

5. Trucco M (2010). Gene-environment interaction in type 1 diabetes mellitus. *Endocrinol Nutr.* 56 Suppl 4:56-9.

CONCLUSION:

The conclusions from the current year of funding are that we demonstrate conclusively the dominant roles of thymic insulin expression in establishing adaptive immune tolerance towards islet beta cells of the pancreas. Ablation of insulin expression in bone marrow derived antigen-presenting cells will not induce autoimmune diabetes. We also identified two types of insulin-expressing stromal cells with antigen-presenting capability in the stroma of secondary lymphoid organs, which might play roles in maintaining peripheral islet beta cell tolerance under central defective conditions.

The So What Section:

What are the implication of this research?

Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are type 1 DM. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. Type 1 diabetes is associated with a high morbidity and premature mortality due to complications. The annual cost from diabetes overall exceeds $100 billion, almost $1 of every $7 dollars of US health expenditures in terms of medical care and loss of productivity.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etiopathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patient well being.

Our first quarterly scientific progress report for the second year of our project (09/28/10 – 12/27/10) described the following:

Background and Significance

Insulin expression was found in both lymph node (LN)-resident stromal cells of non-hemaetopoietic origin and bone marrow (BM)-derived antigen-presenting cells (APCs)[1-3]. Although little is known about the function of insulin expression in LNs, recent studies have shown that TSA-expressing stromal cells can effectively deplete TSA-specific autoreactive CD8+ T cells from the peripheral repertoire[4-6]. As for insulin expression in BM-derived APCs, discordant results have been reported regarding the specific cell subsets. Both (pro)insulin transcripts and proteins were found in human $CD11c^{+}$ dendritic cells (DCs) in the thymus and the peripheral lymphoid tissues[7]. In contrast, Hansenne *et al.* found neither *Ins1* nor *Ins2* transcripts in mouse $CD11c^{high}$ DCs, regardless of their maturation status[8]. Transplantation of BM cells harvested from NOD.*Ins2*$^{+/+}$ mice failed to slow down diabetes progression in NOD.*Ins2*$^{-/-}$ recipients, suggesting that endogenous levels of *Ins2* expression in BM-derived cells of NOD mice cannot restore peripheral tolerance to insulin[9]. However, these data should be interpreted with caution as the authors pointed out that the levels of *Ins2*-expression in spleen and pancreatic LN decrease significantly after weaning (3-4 weeks) in NOD mice[10]. The failure of restoring insulin tolerance might be attributed to the low levels of *Ins2*-expression in the transplanted BM cells. Thus, the role of insulin expression in secondary lymphoid tissues in regulating peripheral tolerance of beta-cells remains elusive.

As reported in the previous quarters, we have generated a number of animal models to investigate the potential role of APC-insulin expression in mediating peripheral tolerance of beta-cells. Recently, we have focused our effort on the CD11c-ΔIns/H2g7 model, as we have shown that insulin expression is restricted to $Aire^{+}CD11c^{int}$ cells of BM-origin in the spleen. In this quarter, we have further characterized this population of cells, and identified them as tolerogenic plasmacytoid dendritic cells (pDCs). In addition, we investigated further the anti-insulin autoimmunity in CD11c-ΔIns/H2g7 mice, and found that although these animals can effectively maintain homeostasis of blood glucose, elevated levels of lymphocyte infiltration into the pancreata were observed, suggesting a partial break down of peripheral tolerogenic mechansims to beta-cells.

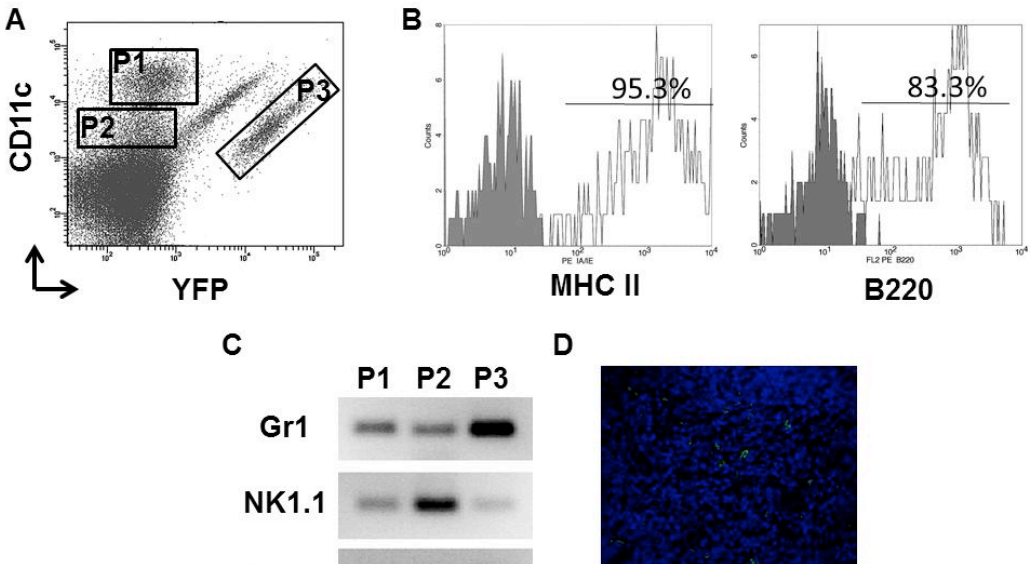
Results of Experiments in Progress

1) Characterization of insulin-expressing antigen presenting cells

The absence of anti-islet autoimmunity in ID-BMC mice prompted us to identify the subset(s) of BM-derived APCs which express insulin. Since it has been shown that *Ins2* expression within the thymus is regulated by the autoimmune regulator (Aire) gene, and that low levels of Aire gene expression are found in BM-derived $CD11c^{+}$ dendritic cells (DCs), we setup to examine whether insulin is also expressed in Aire-expressing cells of BM-origin. Taking advantage of our previous findings that the Aire-Cre transgene can faithfully recapitulate the endogenous Aire gene expression pattern, we crossed the Aire-Cre transgenic mice to reporter RosaYFP mice to generate the Aire-Cre:RosaYFP line, in which all the Aire-expressing cells were positively marked with YFP. FACS analyses showed that about 1-2% of the $CD45^{+}$ splenocytes were YFP-positive (Figure 1A). Of interest, these YFP+ cells expressed intermediate levels of DC marker CD11c, as well as high levels of MHCII and B220 molecules. Combined, this is the molecular signature of the previously described tolerogenic plasmocytoid DCs (pDCs)[56] (Figure 1B).

In addition, another recently identified DC subset, the interferon-g (IFN-g)-secreting natural killer DCs (NKDCs) was also found to express the B220 maker on the surface. To further determine the identity of the $CD11c^{int}$YFP+ population, we separated the $CD11c^{+}$ cells into three populations via FACS: the $CD11c^{high}YFP^{-}$ "conventional" DCs (P1), the $CD11c^{int}YFP^{-}$ DCs (P2) and the $CD11c^{int}$YFP+ "tolerogenic" DCs (P3). RT-qPCRs were performed to examine the expression levels of genes specific to different DC subsets. It was previously shown that Gr-1 is highly expressed in pDCs, whereas NK cell marker NK1.1 is predominantly found in NKDCs. As shown in Figure 1C, high levels of Gr-1 transcripts were found in $CD11c^{int}YFP^{+}$ P3 cells, whereas NK1.1 signal was barely detectable, suggesting that these cells belong to the pDC subset. In contrast, cells in the $CD11c^{int}YFP^{-}$ P2 population expressed higher levels of NK1.1 but lower levels of Gr-1, suggesting that the P2 population was comprised primarily of NKDCs (Figure 1C).

Figure 1. Insulin expression in the spleen is largely restricted to Aire^{+}CD11c^{int}B220^{+} tolerogenic pDCs. A.



FACS analysis of splenocytes isolated from Aire-Cre:RosaYFP mice. Cells were positively selected by anti-CD45 conjugated magnetic beads, followed by staining with anti-CD45 and anti-CD11c antibodies. As shown, CD11c+ cells (gated on CD45+) were separated into three populations: P1, CD11c^{high}YFP^{-}; P2, CD11c^{int}YFP^{-}; P3, CD11c^{int}YFP^{+}. Shown is a representative FACS result from three independent experiments of spleens pooled from 3 animals. **B.** Cells of P3 population was sorted and stained with anti-MHCII and anti-B220 antibodies (solid lines), or IgG controls (filled grey lines). **C.** RT-PCR analysis of expression of genes specific to subsets of DCs. Gr1, pDCs; NK1.1, NKDCs. **D.** Immunohistochemical analysis of spleen sections of Aire-Cre:RosaYFP mice. 5um cryosections were probed with anti-YFP antibody (green).

Histologic examination revealed that YFP^{+} cells were primarily located in the marginal zone, consistent with previous reports (Figure 1D). These findings, in conjunction with our previous RT-qPCR results showing that insulin transcripts were essentially undetectable in either the conventional DCs (P1) or the CD11c^{int}YFP^{-} NKDCs (P2), but were predominantly present in Aire+ pDCs (P3). These data indicate that, unlike thymus and lymph nodes, insulin expressions in the spleen are largely restricted to Aire+ tolerogenic pDCs of BM origin.

2) Characterization of anti-insulin autoimmunity in CD11c-ΔIns/H2g7 mice

As reported previously, CD11c-ΔIns/H2g7 mice can effectively maintain normal blood glucose levels. When challenged with intraperitoneal glucose injections, no difference of glucose metabolism was observed between and littermate controls, suggesting the existence of sufficient islet function. However, consistent with previous reports, the introduction of the diabetes-prone H2g7 MHC alleles to B6 mice (B6/H2g7 congenic) was sufficient to induce low degrees of insulitis in control littermates (Figure 2, top panels), while both the severity and the percentage of islets infiltrated by T cells were significantly increased in pancreata harvested from CD11c-ΔIns/H2g7 mice (Figure 2, lower panels). Specifically, about 50% of islets were infiltrated with CD4^{+} T cells, as compared with less than 20% in controls (53±15% vs. 14±4%, $p<0.05$). Similar levels of islet infiltration were observed for CD8^{+} T cells (46%±6% vs. 16±4%, $p<0.01$). Elevated levels of CD4^{+} and CD8^{+} T cell infiltration in CD11c-ΔIns/H2g7 suggest that insulin-expressing, Aire+CD11c^{int} tolerogenic pDCs might play a role in controlling the activation and expansion of islet-specific CD4^{+} effector and CD8^{+} cytotoxic T cells which have escaped thymic negative selection.

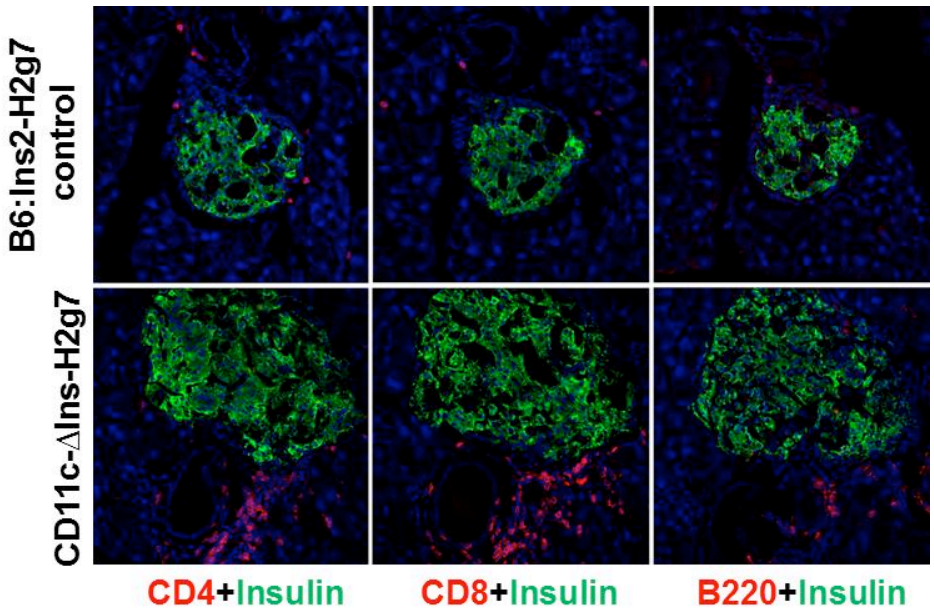


Figure 2. CD11c-ΔIns-H2g7 mice displayed increased levels of islet autoimmunity. Immunohistochemical analyses of pancreata harvested from CD11c-ΔIns/H2g7 mice and non-Cre littermate controls (B6:Ins2/H2g7). Shown are representative consecutive cryosections stained with anti-insulin antibody (green), in conjunction with anti-CD4 (left panels), anti-CD8 (middle panels) and anti-B220 (right panels) antibodies (red).

As one of the potential mechanisms of controlling autoreactive T cell expansion is mediated by the suppressive action of T regulatory (T_{reg}) cells, we examined the presence of T_{reg} cells in both the pancreatic lymph nodes and spleens of CD11c-ΔIns/H2g7 mice. Compared to the littermate controls, no decrease of either percentage or absolute number of T_{reg} cells was observed, suggesting that pDC-insulin mediates peripheral b-cell tolerance primarily through other immune tolerogenic mechanisms (data not shown).

3) CD11c-ΔIns/H2g7 mice developed autoimmune sialadenitis.

Of interest, lymphocyte infiltration was found in salivary glands of CD11c-ΔIns/H2g7 mice, but not in littermate B6/H2g7 controls (Figure 3). Both CD4+ effector T cells and CD8+ cytotoxic T cells were present, indicating the presence of ongoing autoimmune responses targeting salivary glands. We also observed elevated levels of B220+ cells. Further characterization revealed that these cells were negative for B-cell marker CD19, suggesting that they most likely belonged to the B220+ DC subsets. As it was reported previously that insulin transcripts were found at low levels in salivary glands, the unexpected observation of T-cell infiltration into salivary glands suggested that insulin deletion in Aire+ pDCs might also induce tolerance breakdown of salivary glands. These data demonstrated further the essential role of insulin-expression in Aire+ pDCs in mediating peripheral tolerance of insulin-expressing tissues.

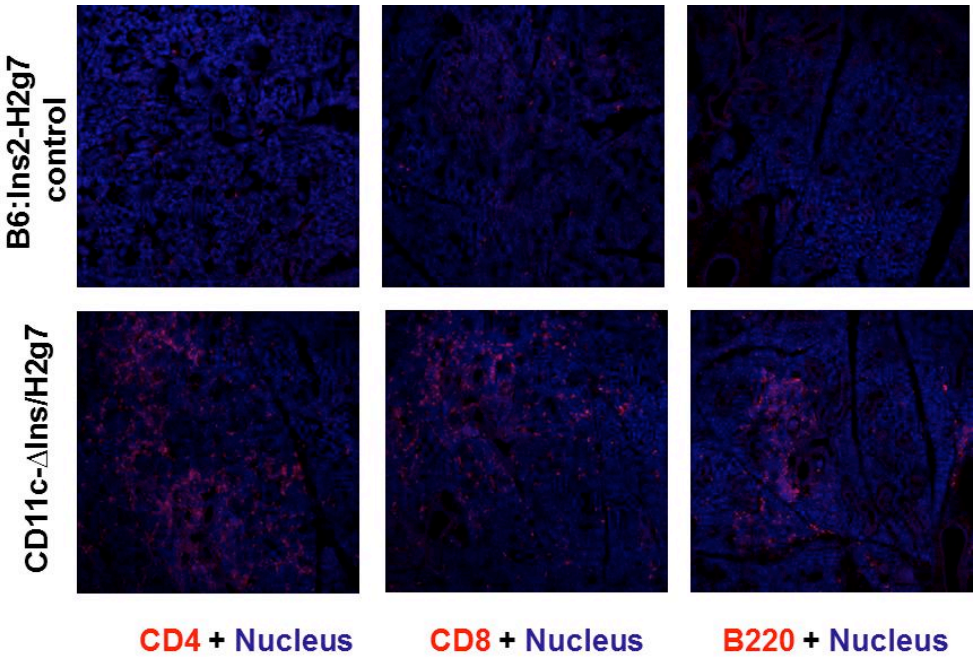


Figure 3. CD11c-ΔIns-H2g7 mice developed autoimmune sialadenitis. Immunohistochemical analyses of salivary glands harvested from CD11c-ΔIns/H2g7 mice and non-Cre littermate controls (B6:Ins2/H2g7). Shown are representative consecutive cryosections stained with anti-CD4 (left panels), anti-CD8 (middle panels) and anti-B220 (right panels) antibodies (red).

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

4) We have identified the insulin-expressing cells as a population of pDCs with the $Aire^{+}CD11c^{int}B220^{+}MHCII^{+}Gr1^{high}NK1.1^{low}$ phenotype. We will further characterize these cells in the next quarter. The ultimate goal is to explore the possibility of using these cells to treat T1D.

5) We found more severe levels of insulitis in pancreata of CD11c-ΔIns/H2g7 mice, compared to B6/H2g7 littermate controls. Our data suggested an essential role of insulin-expression in Aire+ pDCs in mediating the maintenance of peripheral tolerance towards pancreatic b-cells.

6) In addition to insulitis, CD11c-ΔIns/H2g7 mice also developed autoimmune sialadenitis, similar to the type 1 Sjogren syndrome in human. Of note, 20-30% of T1D patients also developed other forms of autoimmune disorders. Further characterization of the autoimmune responses to salivary glands in CD11c-ΔIns/H2g7 mice will help us to delineate the underlying mechanisms of autoimmune disorders associated with T1D.

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Our second quarterly scientific progress report for the second year of our project (12/28/10 – 03/27/11) described the following:

Background and Significance

The essence of adaptive immunity is its capability to effectively distinguish self from non-self and take actions accordingly: eliciting protective immune response to invading pathogens while maintaining immune unresponsiveness to self-antigens under steady state. To establish a self-tolerant, functional T-cell repertoire, developing T-cells must survive both the positive and the negative selections mediated by self-antigen-presenting thymic stromal cells. T-cells fail to establish stable contacts with thymic epithelial cells of the thymic cortex will receive no survival signal and perish by negligence (positive selection), whereas T-cells bearing T-cell receptors (TCRs) with high affinity to self-antigens are considered to be autoreactive and undergo apoptosis within the thymic medulla (negative selection). While it is conceivable that blood-borne self-antigens can be captured and engulfed by the BM-derived APCs and be trafficked back to the thymus, the mechanism of thymic presentation of autoantigens of tissue-specific nature remained elusive until about a decade ago when above-the-noise levels of transcripts of tissue-specific antigens (TSA) were found in mTECs. One of the master regulators of TSA ectopic expression in mTECs is the autoimmune regulator (Aire) gene. Patients with mutations in the Aire gene develop autoimmune polyglandular syndrome I (APS-I), a rare autoimmune disorder affecting multiple organs and tissues, which is also known as autoimmune polyendocrinopathy, candidiasis, and ectodermal dysplasia (APECED). Targeted mutagenesis of the mouse Aire gene partially recapitulated the human disease symptoms – autoimmune responses, manifested as the presence of autoantibodies targeting multiple organs and tissues as well as lymphocyte infiltration, were observed in these animals. Of note, expressions of more than 300 TSAs are significantly down-regulated in mTECs, providing, for the first time, direct experimental evidence linking defective thymic TSA expression with peripheral multiple organ autoimmunity.

However, in most human autoimmune disorders of organ/tissue-specific nature, such as autoimmune diabetes type 1A (T1D) and autoimmune thyroid disease (ATD), one specific organ or tissue is primarily targeted. In addition, autoimmune responses targeting to one (or more) specific TSA(s), such as insulin and thyroglobulin, were often found to be the pivotal driving forces for disease progression. Thus, the broad effects of Aire inactivation on thymic TSA expression and its suggestive roles in mTEC differentiation and maturation render it difficult to study the impact of individual TSA in establishing immunologic tolerance of a specific organ with the Aire-KO animal model.

As reported previously, we have successfully established the ID-TEC animal model in which insulin expression is specifically abrogated in mTECs, but remains intact in pancreatic beta-cells (designated as ID-TEC mouse for insulin-deletion in mTECs). Our results demonstrated conclusively the essential roles of mTEC-insulin expression in mediating central negative selection of insulin-reactive T-cells to establish immune tolerance of insulin-secreting islet beta-cells of the pancreas. However, whether the similar thymic imaging/islet tolerance principle of insulin can be applied to other TSAs remains unanswered.

In this quarter, we succeeded in establishing the Aire-Cre:iDTR model, in which the human diphtheria toxin receptor is expressed exclusively in Aire-expressing mTECs in the thymus. This novel animal model enables us to further evaluate the immunomodulatory roles of mTECs in establishing immune self-tolerance of peripheral organs. Also in this quarter, we have successfully overcome one of the technical obstacles in our attempts to identify and characterize fluorescent molecule labeled T-regulatory cells in transgenic models.

Results

1. Efficient detection of T-regulatory cell-specific FoxP3 proteins in YFP-expressing cells.

As described in our previous Quarterly report, we have identified a population of Aire-expressing APCs in the spleen which display surface markers of tolerogenic plasmacytoid dentric cells (pDCs, $CD11c^{low}$, $MHCII^{+}$,$Gr1^{+}$,$B220^{+}$). Consistently, high levels of insulitis were observed in pancreata of animals in which the mouse Ins2 gene is deleted in CD11c-expressing cells under the $H2^{g7}$ and Ins1-knockout genetic backgrounds, suggesting that insulin-expression in CD11c cells is essential for maintenance of peripheral tolerance of islet beta-cells. Of note, one of the potential peripheral tolerogenic mechanisms mediated

through pDCs is to promote the proliferation and survival of antigen-specific T-regulatory cells. Thus, the ability to track and characterize a specific population of T-regulatory cells, which are fluorescently labeled in vivo, is important to unravel the mechanism of insulin-expressing pDCs in exerting their protective roles of islet autoimmunity.

However, FACS detection of cytosolic or nuclear proteins in fluorescent protein (FP)-expressing cells has been shown to be technically challenging as loss of FP signal is frequently observed when the intracellular staining procedure is used. The common approach to overcome this obstacle is to FACS sort the FP-expressing cells first, followed by intracellular characterization. However such an approach is not only time-consuming, but also impractical when studying rare cells. We evaluated and modified the intracellular staining procedure for simultaneous detection of nuclear proteins and cytosolic YFP molecules, using the nuclear Foxp3 molecule expressed in T-regulatory cells of Vav-Cre:Rosa-YFP reporter mice as a model target.

To identify Foxp3+ T-regulatory cells in Vav-Cre: Rosa-YFP mice, we first stained YFP+ splenocytes with antibodies specific to surface markers of T cells (i.e., CD3 and CD4), and subsequently subjected the cells to the intracellular staining procedure outlined in Figure 1A. To our disappointment, although we were able to stain nuclear Foxp3 proteins efficiently, the YFP signal became essentially undetectable (Figure 1B). Loss of YFP signal could result either from fixative-induced conformation changes of the YFP proteins, or from the loss of cytosolic YFP proteins due to the leakiness of the permeabilized cell membrane. To investigate whether over-fixation is the major causative factor, we shortened the Fixation/Permeabilization (Fix/Perm) buffer treatment from 2 hours to 5 minutes, but still failed to preserve detectable YFP signal (Figure 1C). Indeed, even in the absence of fixatives, we observed significant reduction of cellular YFP signal after only 15 seconds of permeabilization buffer treatment, suggesting that the rapid loss of the fluorescent signal is due to diffusion of YFP molecules out of the permeabilized cell.

To further demonstrate that the leakiness of the permeabilized cell membrane is the cause of the fluorescent signal loss, we took advantage of the eGFP-Foxp3 transgenic mice, in which an eGFP molecule was inserted in-frame to the 5' end of the Foxp3 gene to encode a functional eGFP-Foxp3 fusion protein. One of the major functional components in the permeabilization buffer used is saponin, which can complex with cholesterol to form pores in the cholesterol-rich cytoplasm membrane but leaves the cholesterol-poor nuclear membrane largely intact. The nuclear eGFP-Foxp3 fusion protein should be retained in the nucleus under such treatment and any reduction of eGFP signal should be attributed to the fixative. Splenocytes harvested from the eGFP-Foxp3 mice were treated with Fix/Perm solution overnight, followed by intracellular staining with anti-Foxp3 antibodies. No drastic loss of eGFP signal was observed and the eGFP+Foxp3+ population can be easily identified by FACS analysis (Figure 1D).

It has been shown that pre-fixation of the YFP+ cells with 2% paraformaldehyde is sufficient to effectively preserve the YFP signal, while the efficiency of intracellular staining of FoxP3 is significantly compromised after 30 minutes of paraformaldehyde treatment (data not shown). We speculated that the loss of FoxP3-staining could be caused by over fixation of the cytoplasmic membrane, which impeded the entry of anti-FoxP3 antibody into the cells. To systematically evaluate the effects of fixative pretreatment in intra-nuclear staining, we treated splenocytes harvested from the eGFP-Foxp3 transgenic mice with 2% paraformaldehyde from 15 seconds to 30 minutes, prior to staining of the cells with anti-Foxp3 antibodies. As shown in Figure 2A, pre-fixation from 15 seconds to 2 minutes does not drastically affect the percentage of FOXP3+ cells, whereas more than 5 minutes of paraformaldehyde treatment significantly decreases the antibody staining of the eGFP-Foxp3 fusion proteins in the nucleus.

Figure 1

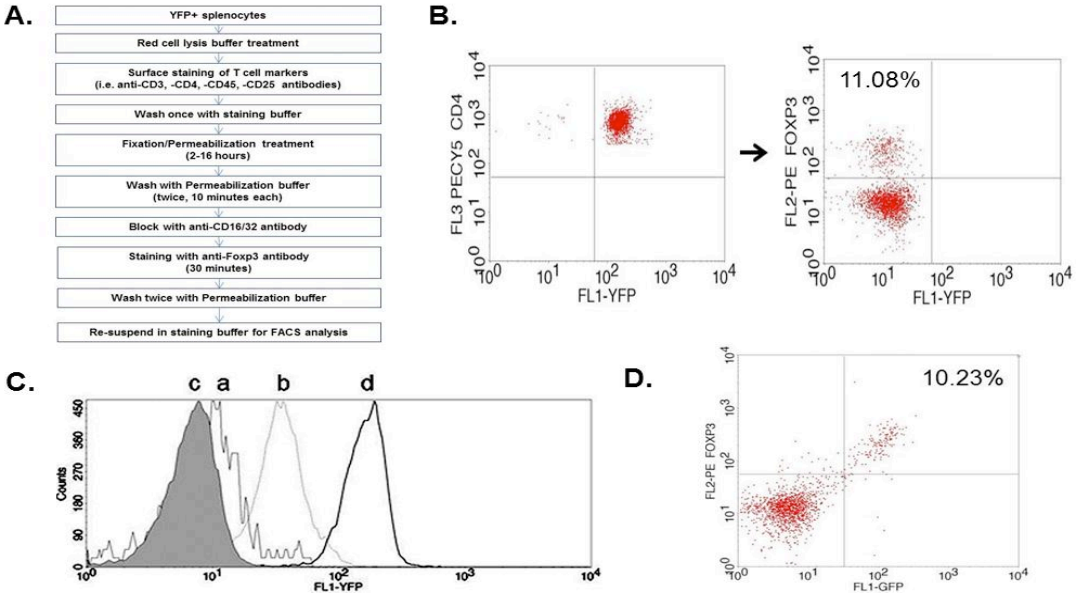

Figure 1. Loss of YFP signal in cells treated with Fixative/Permeabilization reagents used in intracellular staining procedures. A. A step-by-step protocol for intracellular staining of nuclear Foxp3 protein in T regulatory cells. **B.** YFP+, CD4+T cells harvested from Vav-Cre:Rosa-YFP mice were stained intracellularly with anti-Foxp3 antibodies following the procedure outlined in **A**. *Left panel*, before Foxp3 staining; *Right panel*, after Foxp3 staining. **C.** Splenocytes of Vav-Cre:Rosa-YFP mice were treated with Fix/Perm buffer for 5 minutes (**a**), or 15 seconds with permeabilization buffer (**b**). **c**, non-YFP control; **d**, untreated YFP+ T cells. Shown is a representative of FACS result from three independent experiments. **D.** Representative FACS result showing that nearly all the Foxp3 positive CD4+ T cells harvested from eGFP-Foxp3 mice are EGFP positive.

We thus speculated that the success of staining the nuclear FoxP3 molecules while preserving detectable YFP signals might hinge on the optimization of pre-fixation of cytoplasmic membrane to block the leakage of YFP molecules from cytosol, and to preserve the accessibility of the antibody to its nuclear target. To evaluate whether the short pre-fixation treatment is sufficient to block the leakage of the cytosolic YFP proteins, we treated splenocytes harvested from the Rosa-YFP mice with 2% of paraformaldehyde for less than 2 minutes before treating the cells with Fix/Perm buffer. With pre-treatment as short as 15 seconds we were able to detect weak YFP signals. Pre-fixing samples for more than 1 minute was more efficient to achieve a well-defined separation of YFP positive cells from YFP negative controls (Figure 2B). We next proceeded to add the short pre-fixation step to the intracellular staining protocol for Foxp3 detection in Rosa-YFP T regulatory cells. As shown in Figure 2C, a distinct population of CD4+ T cells can be readily identified as double positive for both YFP and Foxp3.

In summary, pre-fixing the cytoplasmic membrane for 1-2 minutes prior to the intracellular staining procedure is sufficient to retain cytosolic YFP proteins within the cells without compromising the antibody detection of cytosolic or nuclear proteins. Such a simple modification of the intracellular staining procedure (Figure 2D) not only allows the usage of FPs as cellular markers for co-localization studies with FACS technology, but may also be applied to intracellular detection of cytosolic proteins to prevent loss of target molecules from membrane leakiness.

Figure 2

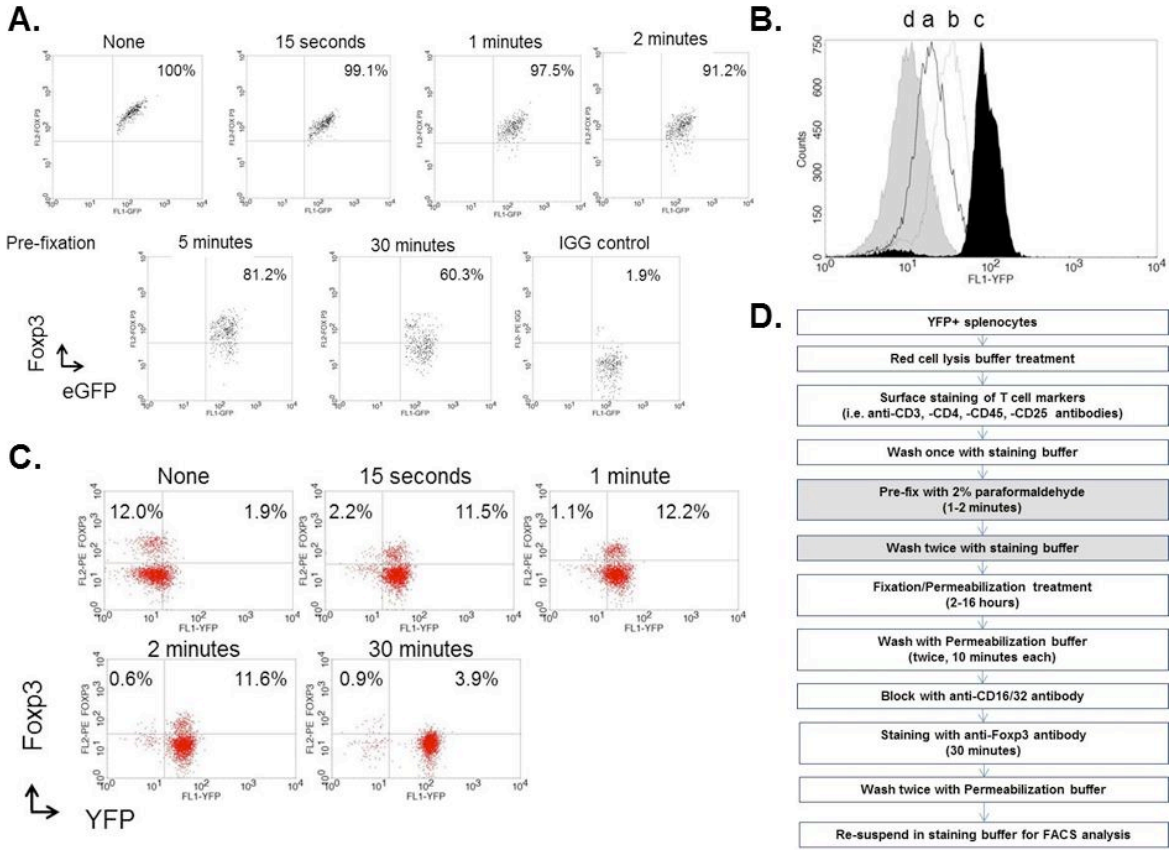

Figure 2. Short pre-fixation prior to intracellular staining is sufficient to retain detectable cytosolic YFP signals. A. Splenocytes harvested from eGFP-Foxp3 mice were stained intracellularly with anti-Foxp3 antibodies and analyzed by FACS (gated on CD4+eGFP+ T regulatory cell population). Percentages of cells with nuclear Foxp3 stained successfully under different pre-fixation conditions are shown. Shown are representative FACS results from five independent experiments. **B.** Splenocytes harvested from Vav-Cre:RosaYFP mice were pre-treated with 2% paraformaldehyde for 15 seconds (**a**), 1 minute (**b**), and 30 minutes (**c**), followed by Foxp3 intracellular staining and FACS analysis for YFP signals. **d**, sample without pre-fixation. **C.** Foxp3-staining of Vav-Cre:RosaYFP CD4+ T-cells pre-treated with 2% paraformaldehyde for various times (shown on top). Percentages of CD4+ T cells positive for both Foxp3 and YFP are shown on the upper right corners. Shown are representative results from five independent experiments. **D.** Modified intracellular staining protocol for nuclear Foxp3 detection. Steps in grey boxes were added to the original procedure in Figure 1A. For other cytosolic or nuclear proteins, optimization of the pre-fixation time will be required.

2. Establishing the Aire-Cre:iDTR animal model.

Taking advantage of the fact that the Aire-Cre transgene can truthfully recapitulate the endogenous Aire gene expression pattern, we crossed the Aire-Cre transgenic animal to Rosa26-iDTR mouse, in which the human diphtheria toxin receptor (DTR) gene, together with a floxed transcriptional blocking cassette at its 5' end, is site-specifically inserted at the Rosa26 locus. Action of the Cre-recombinase will delete the transcriptional blocking cassette from the genome and place the human DTR gene under the direct control of the ubiquitously active Rosa26 promoter. Only in cells transgenically expressing the Cre recombinase gene, human DTR are present at the cell surface; and they are susceptible to diphtheria toxin (DT)-mediated apoptotic cell death. Thus, in Aire-Cre:iDTR mice, DT-injection would, in theory, effectively eliminate mTECs in the thymus, as well as other Aire-expressing APCs in the periphery.
We successfully obtained a number of Aire-Cre:iDTR mice during this quarter. In our initial characterization of Aire-Cre:iDTR mice injected with DT, lymphocyte infiltration in multiple organs was observed, indicating loss of immune tolerance to self in response to mTEC deletion. Of note, these animals were treated at approximately 8-weeks of age, when T-regulatory cell and tolerogenic DC-mediated peripheral tolerogenic mechanisms were well-established, further indicating the dominant roles of central immune organ, the thymus, in mediating immune self-tolerance.

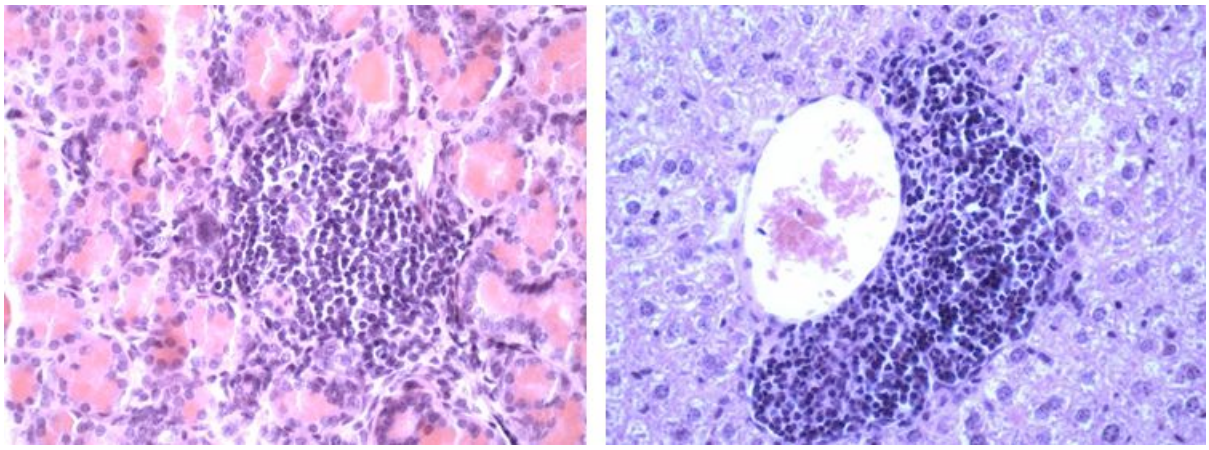

Figure 3. Multiple organ autoimmunity induced by DT-mediated mTEC-depletion. 8-week old Aire-Cre:iDTR mice injected with DT intramuscularly (i.m. at 5ng/kg body weight) for 2 consecutive days. Animals were sacrificed 4-weeks later and examined histologically (H&E). Left panel, salivary glands. Right panel, liver.

In summary, we have successfully developed an intracellular staining protocol which enables us to examine the presence of nucleic proteins, such as T-regulatory specific protein Foxp3 in a specific population of cells, while preserving their cytosolic YFP markers. In addition, we have established an inducible animal model, which will enable us to further unravel the essential roles of Aire-expressing cells in establishing and maintaining immune self-tolerance. Further characterization of these animal models will facilitate our understanding the etiopathogenesis of not only type 1 diabetes, but other autoimmune disorders as well.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

1) We have previously identified a population of insulin-expressing, Aire+ antigen-presenting cells (APCs) in the spleen which express a number of surface markers similar to pDCs ($MHCII^+$, $CD11c^{low}$, $B220^+$, $Gr1^+$). In the next quarter, we will use both molecular and cell biology techniques to further characterize these cells. In addition, we will start to explore the therapeutic potential of these insulin-expressing, Aire+, APCs by transplanting these cells, either alone or together with $FoxP3^+eGFP^+$ T-regulatory cells into prediabetic ID-TEC pups. Diabetes incidence and progression will be monitored. As well, the ability of these APCs to home to the inflammatory pancreases, as well as secondary lymphoid organs of the ID-TEC pups will be examined. These results will be the first step for us to design more sophisticated gene therapy protocols to prolong the survival and efficiency of these tolerogenic insulin-expressing, Aire+ APCs in the future.

2) The establishment of the Aire-Cre:iDTR animal model enables us to investigate the essential roles of Aire-expressing cells in establishing self-tolerance at both central and peripheral levels. In the next quarter, we will examine the function of Aire-expressing, mTECs within the thymus for T-regulatory cell developments. In addition, the roles of Aire+ APCs in the peripheral lymphoid organs in maintaining T-regulatory cell homeostasis will be examined by a bone marrow transplantation model. These results will be instructive to develop therapeutic protocols to prolong the survival and efficiency of islet-specific T-regulatory cells to prevent autoimmune diabetes progression in our ID-TEC model.

In the third quarterly scientific progress report (03/28/10 - 06/27/11) of year 02, we reported on our cumulative results.

Background and Significance

The essence of adaptive immunity is its capability to effectively distinguish self from non-self and take actions accordingly: eliciting protective immune response to invading pathogens while maintaining immune unresponsiveness to self-antigens under steady state. To establish a self-tolerant, functional T-cell repertoire, developing T-cells must survive both the positive and the negative selections mediated by self-antigen-presenting thymic stromal cells. T-cells fail to establish stable contacts with thymic epithelial cells of the thymic cortex will receive no survival signal and perish by negligence (positive selection), whereas T-cells bearing T-cell receptors (TCRs) with high affinity to self-antigens are considered to be autoreactive and undergo apoptosis within the thymic medulla (negative selection). While it is conceivable that blood-borne self-antigens can be captured and engulfed by the bone marrow (BM) -derived antigen presenting cells (APCs) and be trafficked back to the thymus, the mechanism of thymic presentation of autoantigens of tissue-specific nature remained elusive until about a decade ago when above-the-noise levels of transcripts of tissue-specific antigens (TSA) were found in medullary thymic epithelial cells (mTECs). One of the master regulators of TSA ectopic expression in mTECs is the autoimmune regulator (Aire) gene. Patients with mutations in the Aire gene develop autoimmune polyglandular syndrome I (APS-I), a rare autoimmune disorder affecting multiple organs and tissues, which is also known as autoimmune polyendocrinopathy, candidiasis, and ectodermal dysplasia (APECED). Targeted mutagenesis of the mouse Aire gene partially recapitulated the human disease symptoms – autoimmune responses, manifested as the presence of autoantibodies targeting multiple organs and tissues as well as lymphocyte infiltration, were observed in these animals. Of note, expressions of more than 300 TSAs are significantly down-regulated in mTECs, providing, for the first time, direct experimental evidence linking defective thymic TSA expression with peripheral multiple organ autoimmunity.

However, in most human autoimmune disorders of organ/tissue-specific nature, such as autoimmune diabetes type 1A (T1D) and autoimmune thyroid disease (ATD), one specific organ or tissue is primarily targeted. In addition, autoimmune responses targeting to one (or more) specific TSA(s), such as insulin and thyroglobulin, were often found to be the pivotal driving forces for disease progression. Thus, the broad effects of Aire inactivation on thymic TSA expression and its suggestive roles in mTEC differentiation and maturation render it difficult to study the impact of individual TSA in establishing immunologic tolerance of a specific organ with the Aire-KO animal model.

T1D is an autoimmune disease, in which the insulin-secreting pancreatic beta cells are targeted and destroyed by the body's own immune system. Although intensive insulin therapy and tight glycemia control can effectively prevent the devastating secondary complications, as demonstrated by DCCT and other large scale clinical studies, hypoglycemia incidence remains as the major adverse event leading to increase of morbidity and mortality in T1D patients. Of note, numerous researches have shown that even residual islets in T1D patients can significantly lower the risk of severe hypoglycemic incidence and can help to achieve better glycemic control. Thus, developing islet antigen-based immune therapies to restore islet immune tolerance is clinically important to prevent high-risk individuals from developing T1D and to halt islet destruction in recent onset T1D patients.

Our research has been focusing on the immunomodulatory roles of islet autoantigen expression in immune cells in establishing/maintaining islet tolerance. Using the insulin-deleted thymic epithelial cells (ID-TEC) mouse model (Fan et al. ***The EMBO Journal*** 28:2812, 2009), we have demonstrated conclusively the essential roles of mTEC-insulin expression in mediating central negative selection of insulin-reactive T-cells in establishing a T-cell repertoire tolerant to islet beta cells of the pancreas. In addition, we were able to identify a population of hematopoietic lineage-derived Aire-expressing antigen presenting cells (APCs) in the spleen which express surface markers shared by tolerogenic plasmacytoid dentric cells (pDCs, $CD11c^{low}$, MHC Class II^{+},$Gr\text{-}1^{+}$,$B220^{+}$). Notably, insulin transcripts were predominantly found in this population of Aire+ cells. Furthermore, deletion of insulin-expression in CD11c-expressing cells with the CD11c-Cre transgene resulted in elevation of insulitis in animals with defective central negative selection (carrying diabetes-prone $H2^{g7}$ MHC), suggesting a role of insulin-expression in CD11c cells in maintaining peripheral tolerance of islet beta cells.

In an effort to unravel the mechanism of insulin-expressing pDCs in exerting their protective roles of islet autoimmunity, we further characterized the Aire$^+$ pDC-like cells in the spleen in this quarter. In addition, we further refined the intracellular staining protocol for effective detection of fluorescent molecule labeled T-regulatory cells.

3. Further characterization of Aire$^+$ APCs in the spleen.

To further characterize the Aire+, pDC-like cells described previously, we FACS sorted the YFP$^+$ cells (representing Aire$^+$ cells) from Aire-Cre:RosaYFP mice and subjected them to surface marker analysis. To our surprise, only about 5-6% of all YFP+ cells express mPDCA-1, one of the pDC-specific surface markers routinely used to define the pDC population. Thus, the Aire$^+$ cells identified in our model might not represent APCs of one specific lineage, rather, Aire-positivity, in conjunction with other surface markers, such as B220, MHCII, Gr-1 and CD11c intermediate/low, might actually reflect the common molecular property of various tolerogenic APCs. As reported previously, the level of MHC Class II and costimulatory molecules CD80 and CD86 expression are indicators of a maturation status of APCs: lowly expressed in immature DCs and tolerogenic pDCs at resting stage, but significantly up-regulated upon activation and antigen exposure. To further characterize the properties of Aire$^+$ APCs isolated from the spleen, we used magnetic bead technology to separate YFP$^+$ cells into two groups based on their levels of MHC Class II and costimulatory molecule expression. YFP$^+$ cells predominantly belong to the MHC Class II^{high} group, whereas less than 0.5% expresses low levels of MHC Class II and co-stimulatory molecules. As shown in Figure 1A, about 40-50% of YFP$^+$MHC Class II^{low} cells express high levels of pDC-specific marker, mPDCA-1, suggesting that they are resting pDCs. In contrast, only about 5% of YFP$^+$MHC Class II^{high} cells are positive for mPDCA-1. The levels of mPDCA-1 expression in mPDCA1$^+$YFP$^+$MHC Class II^{high} cells were significantly lower than those of mPDCA1$^+$YFP$^+$MHC Class II^{low} and mPDCA1$^+$YFP$^-$ cells (Figure 1B). Thus, down-regulation of mPDCA-1 expression might be the result of YFP$^+$ APC activation. As YFP$^+$ APCs ectopically express and present self-antigens, such as insulin, it is conceivable that they might interact predominantly with T-cells bearing autoantigen-reactive TCRs, to induce anergy of self-reactive effector T-cells or to promote the survival/proliferation of T-regulatory cells. Indeed, we have observed a positive correlation between the numbers of T-regulatory cells and the numbers of YFP$^+$ APCs in aged animals (data not shown), suggesting a role of YFP$^+$ APCs in supporting T-regulatory survival.

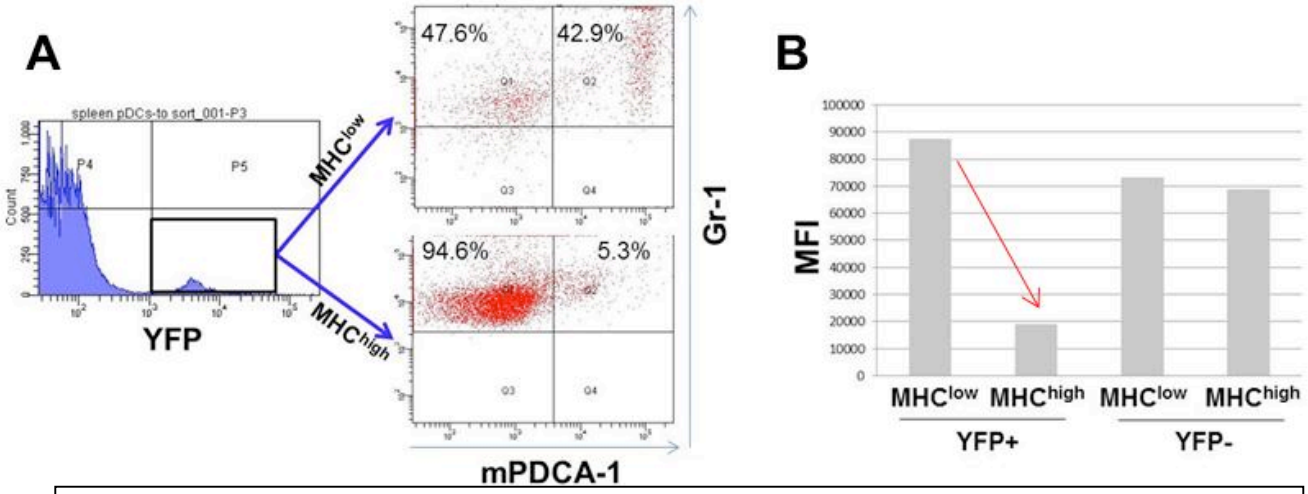

Figure 1. Characterization of YFP+ cells harvested from Aire-Cre:RosaYFP spleens. A. Representative FACS data showing that the YFP$^+$ cells are predominantly mPDCA-1 negative. **B.** In mPDCA-1 positive populations, activated YFP$^+$MHC^{high} cells express significant lower levels of mPDCA-1, in comparison to MHC^{low}YFP$^+$ or YFP$^-$ cells. MFI = mean fluorescent intensity.

4. Optimization of intracellular staining protocol to examine YFP$^+$ T-regulatory cells.

In the previous quarter, we have developed a novel FACS method which enables us to intracellularly stain the nucleic FoxP3 proteins while preserving detectable levels of cytosolic YFP proteins for lineage identification. The success of procedure hinged on the optimization of pre-fixation of cytoplasmic membrane to block the leakage of YFP molecules from cytosol, and to preserve the accessibility of the antibody to its nuclear target. We have shown that a short prefixation with 2% of paraformaldehyde for 2 minutes fits the required fine balance. However, such a short time window of pretreatment might not be suitable for staining a large number of samples simultaneously; and the high concentration of paraformaldehyde fixative used rendered it hard to achieve precise control over the prefixation step. To overcome these problems and to optimize the protocol for broader use, we developed a formula to quantify the effect of prefixation on YFP+ cells undergoing intracellular staining procedure: Prefixative Factor (PF) = % of paraformaldehyde x prefixative time (minute). As shown in the Table below, four different concentrations of paraformaldehyde were systematically evaluated.

Time (min)/**PF**	A	B	C	D
Final conc. of paraformaldehyde	2%	1%	0.5%	0.01%
1	30 min/60	30 min/30	30 min/15	30 min/0.3
2	15 min/30	15 min/15	15 min/7.5	15 min/0.15
3	5 min/10	5 min/5	5 min/2.5	5 min/0.05
4	2 min/4	2 min/2	2 min/1	2 min/0.02
5	1min/2	1min/1	1min/0.5	1min/0.01

As shown in Figure 2A and highlighted in the Table above, PF≥4 ensures sufficient fixation of the plasma membrane to preserve enough cytosolic YFP proteins for detection. Since over fixation of the plasma membrane prevents the entrance of anti-FoxP3 antibodies into the cells (shown previously), we projected that 4≤PF≤20 would represent the optimal conditions for intracellular staining of YFP+ cells (Gray area in Figure 2A). Indeed, we were able to stain FoxP3 efficiently in YFP+ cells under these conditions (Figure 2B and 2C and data not shown).

Summary

In this quarter, we characterized further Aire+ tolerogenic APCs in our Aire-Cre:RosaYFP animal models. Our results suggest that it is rather unlikely that these cells belong to one specific APC lineage, but rather, Aire-positivity represents the tolerogenic properties of self-antigen presenting APCs. As most of these cells express high levels of MHCII and costimulatory molecules, they might actively present autoantigens to T-regulatory cells to promote the homeostasis.

In addition, we have systematically examined and optimized the conditions for intracellularly staining of FoxP3 in YFP+ cells. The protocol enables us to trace the origins of FoxP3+ cells in adoptive T-cell transfer animal models, which will facilitate our efforts to understand the underlying mechanisms to maintain homeostasis of T regulatory cells under physiological and pathologic situations.

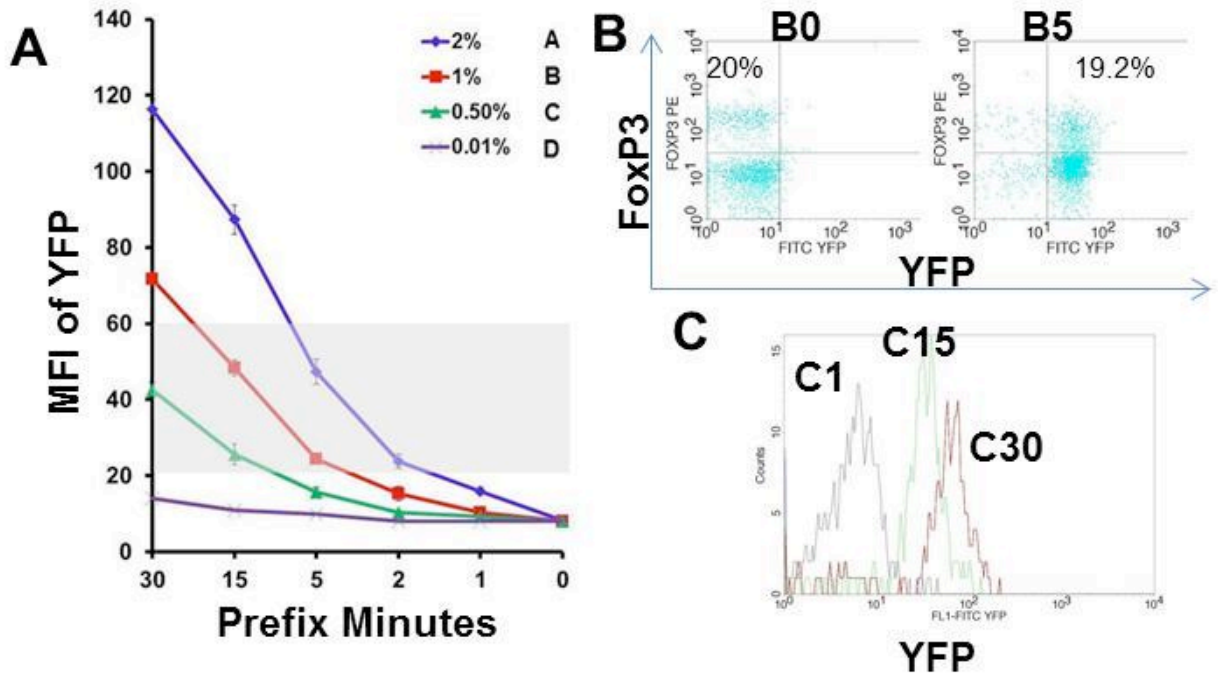

Figure 2. Intracellular staining of nucleic FoxP3 proteins in YFP+ cells. A. Preservation of YFP signals were evaluated in YFP+ cells prefixed with four different concentration of paraformaldehyde (**A**, 2%; **B**, 1%; **C**, 0.5% and **D**, 0.01%) for the designated periods of times. MFI above 20 usually provides sufficient separation of the YFP signals from the untreated controls. The experiments were repeated three times. **B.** Representative FACS results showing the effective detection of FoxP3 proteins without compromising of YFP signals. **B0** and **B5**, prefix with 1% paraformaldehyde for 0 and 5 minutes, respectively. The numbers in the FACS plots show the % of T regulatory cells present in the CD4+ T-cell population. As shown, prefixation under the B5 condition did not compromise FoxP3 staining. The experiments were repeated three times with similar results. **C.** Preservation of YFP signals in cells intracellularly stained for FoxP3. YFP+ samples were prefixed with 0.5% paraformaldehyde for 1 (**C1**), 15 (**C15**) and 30 (**C30**) minutes, followed by FoxP3 staining. Representative FACS results were gated on CD4+FoxP3+ cells. Shown are representative results of three independent experiments.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

3) We have previously identified a population of insulin-expressing, Aire+ antigen-presenting cells (APCs) in the spleen which express a number of surface markers similar to pDCs ($MHCII^{+}$, $CD11c^{low}$, $B220^{+}$, $Gr1^{+}$). In the next quarter, we will use both molecular and cell biology techniques to further characterize these cells. In addition, we will start to explore the therapeutic potential of these insulin-expressing, Aire+, APCs by transplanting these cells, either alone or together with $FoxP3^{+}eGFP^{+}$ T-regulatory cells into prediabetic ID-TEC pups. Diabetes incidence and progression will be monitored. As well, the ability of these APCs to home to the inflammatory pancreases, as well as secondary lymphoid organs of the ID-TEC pups will be examined. These results will be the first step for us to design more sophisticated gene therapy protocols to prolong the survival and efficiency of these tolerogenic insulin-expressing, Aire+ APCs in the future.

4) The establishment of the Aire-Cre:iDTR animal model enables us to investigate the essential roles of Aire-expressing cells in establishing self-tolerance at both central and peripheral levels. In the next quarter, we will examine the function of Aire-expressing, mTECs within the thymus for T-regulatory cell developments. In addition, we will examine the roles of Aire+ APCs played in the peripheral lymphoid organs, in maintaining T-regulatory cell homeostasis, using a bone marrow transplantation model. These results will be instructive to develop therapeutic protocols to prolong the survival and efficiency of islet-specific T-regulatory cells to prevent autoimmune diabetes progression in our ID-TEC model.

5) We will then continue to characterize the Aire-Cre:iDTR animal model developed in the lab, which have shown autoimmune responses targeting multiple organs upon DT-mediated depletion of Aire-expressing cells. Specifically, we will examine the affected organs systematically and investigate the underlying mechanisms of such loss of self-tolerance.

6) The golden standard to define tolerogenic APCs is to demonstrate their capabilities to: 1) inhibit autoreactive T-cell activation upon antigen stimulation, and/or 2) promote survival/proliferation of T-regulatory cells. In the next quarter, we will setup *in vitro* assays to examine whether Aire+ splenocytes possess these tolerance-induction properties. Furthermore, cytokine secretion profiles upon stimulation will also be examined, focusing on those that are essential for tolerance inductions, such as TGF-beta and IL-10. In addition, we will continue to investigate their potential therapeutic function in halting the progression of islet-autoimmunity in prediabetic ID-TEC pups.

In the fourth quarterly scientific progress report (06/28/11 - 09/27/11) of year 02, we now report on our new and cumulative results.

The establishment of the Aire-Cre:iDTR animal model enables us to investigate the essential roles of Aire-expressing cells in establishing self-tolerance at both central and peripheral levels. We then examined the function of Aire-expressing, mTECs within the thymus for T-regulatory cell developments. In addition, we examined the roles of Aire+ APCs played in the peripheral lymphoid organs, in maintaining T-regulatory cell homeostasis, using a bone marrow transplantation model. These results are instructive to develop therapeutic protocols to prolong the survival and efficiency of islet-specific T-regulatory cells to prevent autoimmune diabetes progression in our ID-TEC model.

Developing an intracellular staining protocol for efficient detection of nuclear proteins in YFP-expressing cells

In the previous quarter, we have systematically examined and optimized the conditions for intracellularly staining of T regulatory cell specific transcription factor FoxP3 in YFP+ cells. Our results indicate that the success of simultaneous detection of cytoplasmic YFP proteins and FoxP3 is hinged on the combined effect of effect of prefixation time and concentration of the fixative used. We defined the value of prefixation time (minutes) x % of fixative as prefixation factor (PF), and concluded that while PF≥4 is required to retain enough YFP proteins for flow cytometry (FCM) analysis, P≥30 is detrimental to FoxP3 staining. Since various intracellular antigens may differ substantially in their retention and susceptibility to Fix/Perm treatment, we also examined the nuclear expression of *helios*, a member of nuclear expressed transcription factor of the Ikaros family, in YFP+ splenocytes to demonstrate the potential broad application of our intracellular staining protocol. As shown in Figure 1, effective detection of both Helios and YFP were achieved under conditions similar to FoxP3/YFP staining. Thus, PFs range from 4 to 20 represents the optimal conditions to obtain efficient nuclear protein staining, and well separation of the YFP signals from the background. It is conceivable that the optimal PF will vary in different models; for cells with weak fluorescent signals, a higher PF (10-20) could be preferred, and vice versa. Nevertheless, the simple modification of the intracellular staining procedure described above reliably enables the usage of cytoplasmic FPs as cellular markers for co-localization studies with FCM technology.

Direct contacts between FoxP3+ cells and Aire-expressing stromal cells in secondary lymphoid organs.

We have demonstrated previously that organ-specific self-antigens, such as insulin and islet autoantigen 69 (ICA69), are lowly expressed not only in the thymus, but also present in secondary lymphoid organs. While our ID-TEC animal model have demonstrated conclusively the essential roles of islet autoantigen expression within the thymus in establishing immune tolerance of pancreatic beta cells, the roles of islet antigen production in secondary lymphoid organs remain elusive. To answer these questions, we have developed Vav-Ins and CD11c-Ins animals, in which insulin expression are specifically abrogated in bone marrow-derived antigen presenting cells (APCs) and CD11c-expressing dendritic cells (DCs), respectively. As reported previously, our data suggest that islet autoantigen expression in secondary lymphoid organs play important maintenance roles in restricting the clonal expansion of insulin-reactive T cells in the periphery.

As one potential mechanism for autoreactive T-cell inhibition is through the dominant suppressive activity of T regulatory (Treg) cells. Either derived from the thymus or converted from CD4+ effector T cells, these cells comprise of 10-20% of CD4+ T-cell population and express the transcription factor FoxP3. Elimination of FoxP3+ Treg cells through genetic engineering or antibody based depletion will

lead to unchecked proliferation of effector T-cells, resulting in lymphocyte infiltration into multiple organs and tissues. Interestingly, Treg cells are self-reactive (with cognate autoantigens) and undergo constant turnover and proliferation. However, the underlying mechanism for maintaining Treg cell homeostasis is still largely unknown at present. Based on these findings, **we hypothesized that the Aire- expressing APCs in the secondary lymphoid organs, play essential roles in Treg cell homeostasis in the periphery, by directly presenting organ-specific autoantigens to Treg cells to drive their proliferation.** As the first step to test this hypothesis, we took advantage of our previous findings that the Aire-Cre transgene can faithfully recapitulate the endogenous Aire gene expression pattern, and labeled all Aire-expressing cells with EYFP by generating the Aire-Cre:Rosa26R-EYFP (Aire-YFP) reporter mice. Both spleen and lymph nodes were harvested from the Aire-YFP reporter mice and were subjected to immunohistochemical analysis of Foxp3+ Treg cells and Aire-expressing tolerogenic DCs. As shown in Figure 2 (*top panels*), both Foxp3+ Treg cells and Aire-expressing DCs were localized in the marginal zone areas (MZ), adjacent to the follicular dendritic zone (FDC) of both the spleen and the lymph nodes. Furthermore, cell-cell contacts between Aire-expressing cells and Treg cells were observed (Figure 2, arrows in *lower panels*), suggesting the existence of immunologic cross-talks between the two cell types.

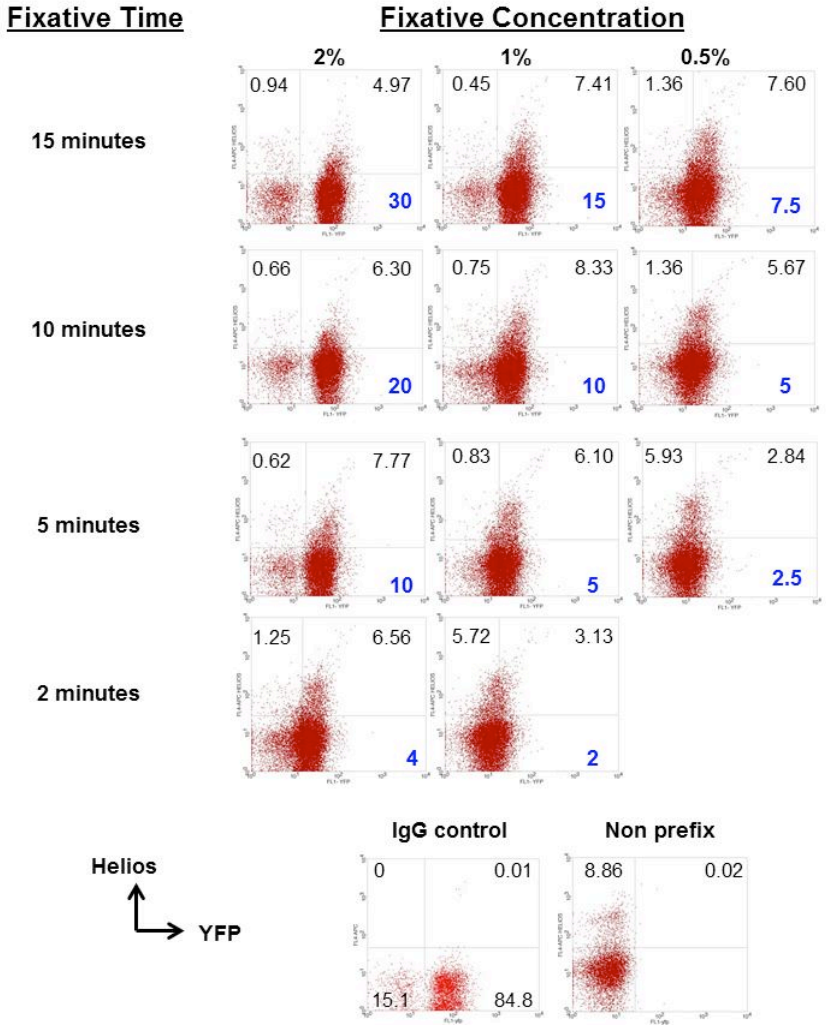

Figure 1. Flow cytometric detection of transcription factor Helios expression in YFP+ splenocytes. Splenocytes harvested from Vav-Cre:RosaYFP were pre-fixed with 0.5%, 1% and 2% paraformaldehyde for the times shown. Percentages of splenocytes positive for both Helios and YFP are shown on the upper right corners. Shown are representative results from two independent e xperiments. The blue numbers in each panel represents the PF factors under each condition. As shown, pre-fixation with PF range from 4-20 allows effectively staining of Helios, while preserving above background levels of YFP signals.

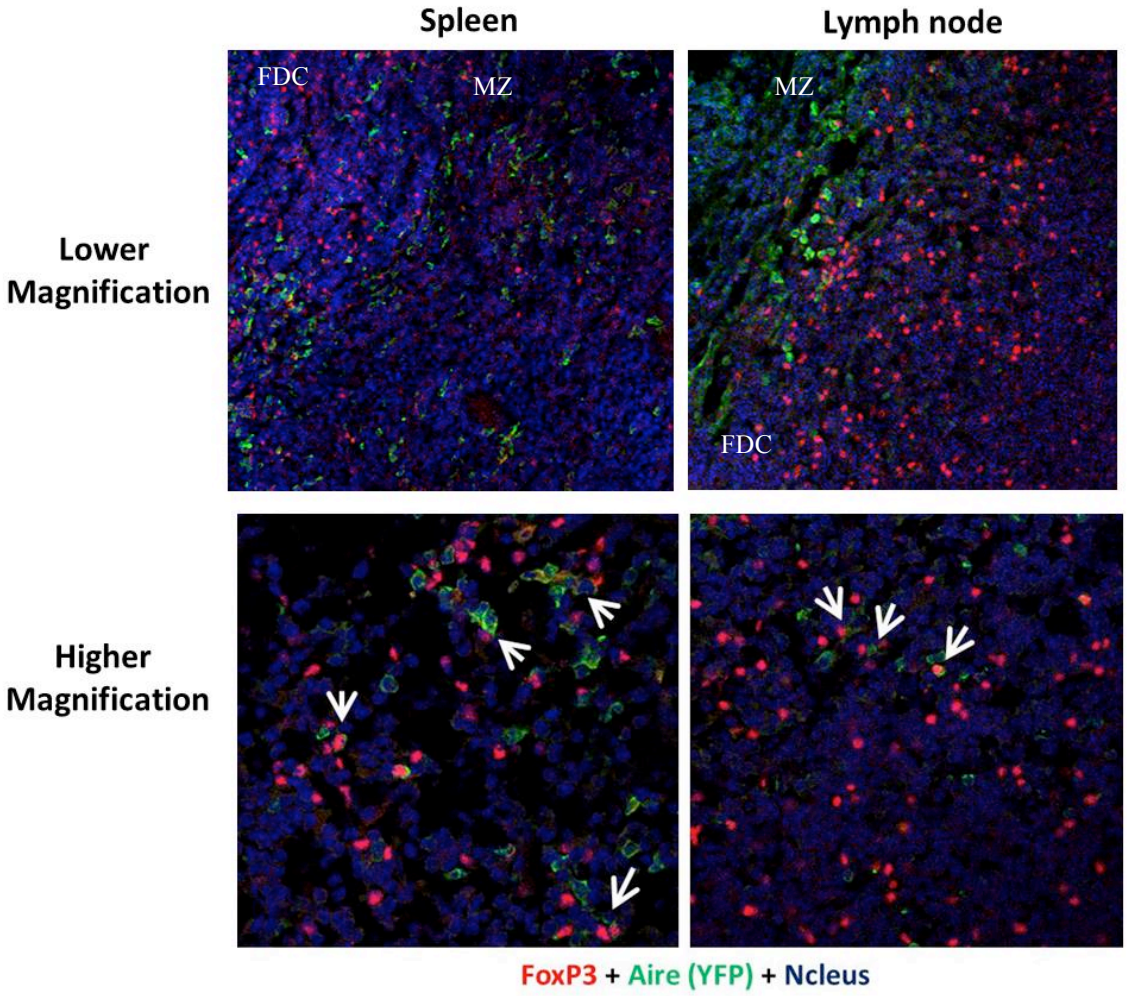

Figure 2. Immunohistochemical analysis of direct cellular interaction between FoxP3+ Treg cells and Aire-expressing tolerogenic DCs. Spleens and lymph nodes were harvested from Aire-Cre:Rosa26R-EYFP mice and fixed with 4% paraformaldehyde for 3 hours at 8^{o}C. Cryosections of 7um were co-stained with anti-FoxP3 (red) and anti-YFP (green) antibodies. Top Panel: low magnification images showing that Aire-expressing cells (green) are predominantly present in the marginal zone (MZ) area adjacent to the follicular dendritic zone (FDC) in both the spleen (Left panel) and the lymph nodes (Right panel). Top Panel: higher magnification images showing the existence of direct interactions between the Foxp3+ Treg cells and Aire-expressing DCs. Arrows indicate the close contacts between Tregs and Aire+DCs in both the spleen and the lymph nodes.

Use additional pages to present a brief statement of plans or milestones for the next quarter.

We will continue to characterize the Aire-expressing cells in secondary lymphoid organs. Specifically, we will systematically examine the expression of islet-specific genes in both the CD45+Aire+ splenocytes and the CD45-Aire+ stromal cells of the lymph nodes. In addition, their antigen-presenting capabilities and their response under immune stimulating conditions will be investigated in vitro. Furthermore, we are in the process of generating the Aire-Cre: IAb-fl/fl mice, in which the antigen presenting MHC II molecules will be specifically knocked out in Aire-expressing cells. This animal line will help us to demonstrate the essential roles of self-antigen presentation by Aire-expressing tolerogenic DCs in maintaining Treg homeostasis.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have successfully generated and characterized the ID-BMC animal model in which insulin expression is abrogated only in bone marrow derived cells. We further demonstrated that the absence of insulin in bone marrow cells is not sufficient to induce autoimmune diabetes, thus, emphasize the dominant roles of central mechanism in T1D etiolgoy.
2. We have identified two insulin-expressing stromal cell types in secondary lymphoid organs, and we will explore their therapeutic potency to strengthen peripheral tolerogenic mechanism to counteract preexisting faulty thymic negative selection conditions.
3. We have successfully generated ID-DC and ID-DC-H2g7 animals, in which insulin expression is specifically knocked out in both classic and tolerogenic dendritic cells. These animals will allow us to dissect further the interactions between central and peripheral immune mechanisms in establishing/regaining insulin tolerance.
4. We Intracellular staining is a widely used flow cytometry (FCM)-based technique to detect the xpression of cytosolic/nucleic antigens. However, intracellular staining of cells expressing cytosolic fluorescent rotein (FP) markers was proven to be problematic as significant loss of the FP-signal was routinely observed. Using splenocytes harvested from mice constitutively expressing the enhanced yellow fluorescent proteins (YFP) as a model, we modified the widely used intracellular staining protocol and successfully achieved simultaneous detection of both the nuclear proteins and YFP in T-regulatory cells. The improved protocol can be used to perform antibody-based intracellular characterization of FP-labeled target cells, while maintaining their fluorescent reporter signals for easy tracing and identification.

REPORTABLE OUTCOMES:

Manuscripts (5 publications)

1. Lu C, Kumar PA, Fan Y, Sperling MA, Menon R: A novel effect of GH on macrophage modulates macrophage-dependent adipocyte differentiation. **Endocrinology** 151(5): 2189, 2010.
2. Perdomo G, Kim DH, Zhang T, Qu S, Thomas EA, Toledo FG, Slusher S, Fan Y, Kelley DE, Dong H: A role of apolipoprotein D in triglyceride metabolism. **Journal of Lipid Research** 51: 1298, 2010.
3. Mavalli M, DiGirolamo D, Fan Y, Riddle R, Campbell K, Sperling M, Frank S, Bamman M, Clemens T: Distinct growth hormone receptor signaling modes control skeletal muscle development and insulin sensitivity. Manuscript accepted for publication in the *Journal of Clinical Investigation*. **J Clin Invest.** doi:10.1172/JCI42447, 2010.
4. Trucco M: Beta-cell regeneration: from science fiction to challenging reality. **Pediatr Diabetes** 11(5):292, 2010.
5. Grupillo M, Lakomy R, Geng X, Styche A, Rudert WA, Trucco M, Fan Y: An improved intracellular staining protocol for efficient detection of nuclear proteins in YFP-expressing cells. **BioTechniques**, In press, 2011.

CONCLUSION:

The conclusions from the current year of funding are that we demonstrate conclusively the dominant roles of thymic insulin expression in establishing adaptive immune tolerance towards islet beta cells of the pancreas. Ablation of insulin expression in bone marrow derived antigen-presenting cells will not induce autoimmune diabetes. We also identified two types of insulin-expressing stromal cells with antigen-presenting capability in the stroma of secondary lymphoid organs, which might play roles in maintaining peripheral islet beta cell tolerance under central defective conditions.

The So What Section:

What are the implication of this research?

Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are type 1 DM. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. Type 1 diabetes is associated with a high morbidity and premature mortality due to complications. The annual cost from diabetes overall exceeds $100 billion, almost $1 of every $7 dollars of US health expenditures in terms of medical care and loss of productivity.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etiopathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patient well-being.

In the first quarterly scientific progress report (09/28/11 - 12/27/11) of year 03, we now report on our new and cumulative results.

1. Characterization of insulin expression in peripheral Aire-expressing cells.

Taking advantage of the Aire-Cre:RosaYFP animal model, we have shown previously that insulin expression in the spleen is restricted to a population of bone marrow-derived MHCII^{+}CD11c^{Int}B220^{+}Gr1^{+} cells, which also express the autoimmune regulator (Aire) gene. To further evaluate the levels of insulin expression in peripheral immune organs, we isolated both splenocytes and islets from collagenase digestion of spleens and pancreata harvested from the Aire-Cre:RosaYFP mice, respectively. Aire-expressing cells were further enriched by FACS sorting of EYFP^{+} cells (P3), as well as EYFP^{-}CD11c^{+} APCs (including both conventional dendritic cells and NKDCs, P1+P2). Insulin mRNA expression levels were examined by real-time RT-qPCR analysis (Figure 1). As shown, the average number of insulin mRNA transcripts in Aire-expressing cells (P3) is about 1-2% that of the house-keeping gene Hprt, and is about 0.001% that of insulin transcripts in pancreatic islets. As there is approximately 10 molecules of Hprt mRNA transcript per cell, Aire-expressing splenocytes have 0.1 insulin transcript per cell, in contrast to islet cells which have an average of 1000-2000 insulin mRNA molecules.

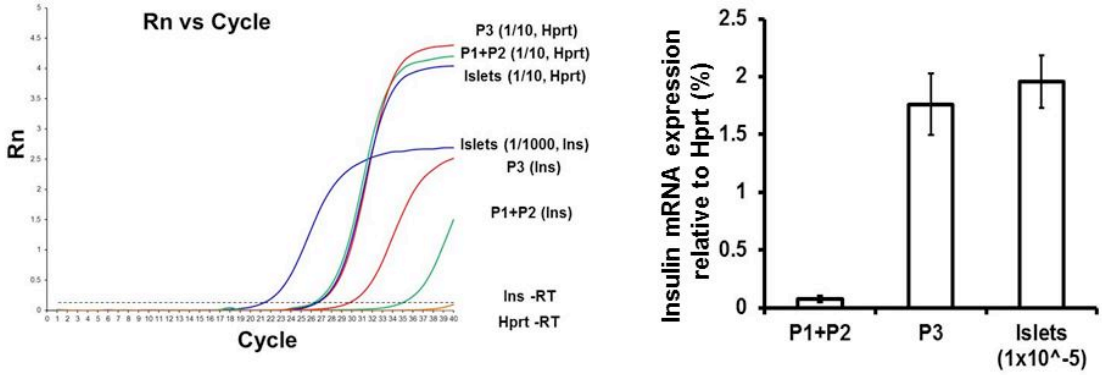

Figure 1. RT-qPCR analysis of insulin expression in Aire-expressing splenocytes (P3). EYFP+ cells were harvested and FACS sorted from a pool of three spleens of Aire-Cre:RosaEYFP mice, and were subjected to RT-qPCR analysis of insulin expression, in comparison to CD11c^{high}/CD11c^{Int}NK1.1^{+}Gr1^{-} antigen presenting cells (P1+P2), and pancreatic islets. *Left panel*, realtime RT-PCR amplification plot shows cycle number versus normalized reporter (Rn) fluorescence. The original islet cDNA samples were diluted 10 and 1000 times before used as input template for the amplification of *Hprt* and *Insulin*, respectively. *Right Panel*, levels of *Insulin* mRNA expression relative to *Hprt*. As shown, the number of insulin transcripts in P3 is approximately 1-2% of that of *Hprt*, and about 0.001% of the pancreatic islets.

2) General physiology of B6^{b}.Vav-ΔIns and B6^{g7}.CD11c-ΔIns mice.

We have shown previously that knocking out insulin in either CD11c+ cells (the B6^{g7}.CD11c-Ins mice**)** or hematopoietic lineage-derived cells (the B6^{b}.Vav-ΔIns mice) will not induce anti-islet autoimmunity, suggesting that insulin expression in bone marrow derived APCs are dispensable for negative selection of insulin-reactive thymocytes. Furthermore, we have demonstrated that insulin expression in CD11c+ cells plays a role in maintaining islet immune tolerance under faulty central negative selection conditions. However, in addition to its immunoregulatory role, insulin production in hematopoietc lineage cells might also function as a tropic factor to support the survival and proliferation of bone marrow cells. To rule out such a possibility, we examined the hemocrit, as well as the metabolites of both B6^{b}.Vav-ΔIns and B6^{g7}.CD11c-ΔIns mice, in comparison to controls (Table 1). Normal hematocrit, metabolites and metabolism were observed, indicating that abrogation of insulin expression in hematopoietc lineage cells does not negatively affect either the hematopoiesis or the overall physiology of the animals.

Table 1. Hematocrit and blood metabolites of Vav-ΔIns and CD11c-ΔIns mice.

	Vav-ΔIns (n=3)	**Control (n=3)**	**CD11c-**ΔIns (n=3)
Hematocrit (% PCV)	44.3±2.1	44.3±2.5	45±4.6
Hemoglobin (g/dL)	15.1±0.7	15.1±0.9	15.3±1.6
Na (mmol/L)	150.3±1.1	149±1	149±1
K (mmol/L)	6.1±0.5	6.1±0.4	6.5±0.5
Cl (mmol/L)	117±2.6	116.7±2.1	116±1
PH	7.31±0.03	7.34±0.01	7.34±0.01
TCO2 (mmol/L)	23.3±1.1	24.7±1.5	27.7±0.6
BUN (mg/dL)	24±1.7	27.7±3.1	33.7±8.5
Glucose (mg/dL)	126±17.4	173.3±17.0	161.7±1.5

3) Efficient depletion of mTECs in Aire-Cre:RosaDTR animal model.

We have shown previously that administration of diphtheria toxin (DT) to Aire-Cre:RosaDTR (Aire-DTR) mice is sufficient to induce multiple organ autoimmunity, manifested by immune cell infiltration into liver, pancreas, salivary glands, tear glands, etc. To further establish that loss of mTEC is the major causative factor, Aire-DTR mice were treated with DT (5ng/g body weight) for three consecutive days, and examined the changes of mTECs each day. Using UEA as a marker for mTECs, we were able to observe a decrease of number of UEA+ cells as early as 24 hours after DT-administration. At 48-hours, both the overall number of UEA+ cells and the intensities of UEA+ were prominently decreased, indicating significant loss of mTECs in the thymus (Figure 2A). Indeed, quantitative examination of UEA+ epithelial areas in thymic sections revealed a gradual loss of mTECs upon DT-treatment (Figure 2B). Furthermore, FCM analysis of the $CD45^{-}g8.8^{+}$ thymic epithelial cells (TECs) showed drastic loss of mTECs 5 days after DT-treatment (Figure 2C). Only ~25% of TECs belong to mTECs, in contrast to controls in which 75% of TECs are present in the medullary region. Since the cortical TECs are largely unaffected by DT administration, these data revealed a 10 times decrease of mTEC numbers in the treated Aire-DTR mice. Thus, DT-administration can effectively induce apoptosis of mTECs in Aire-DTR mice.

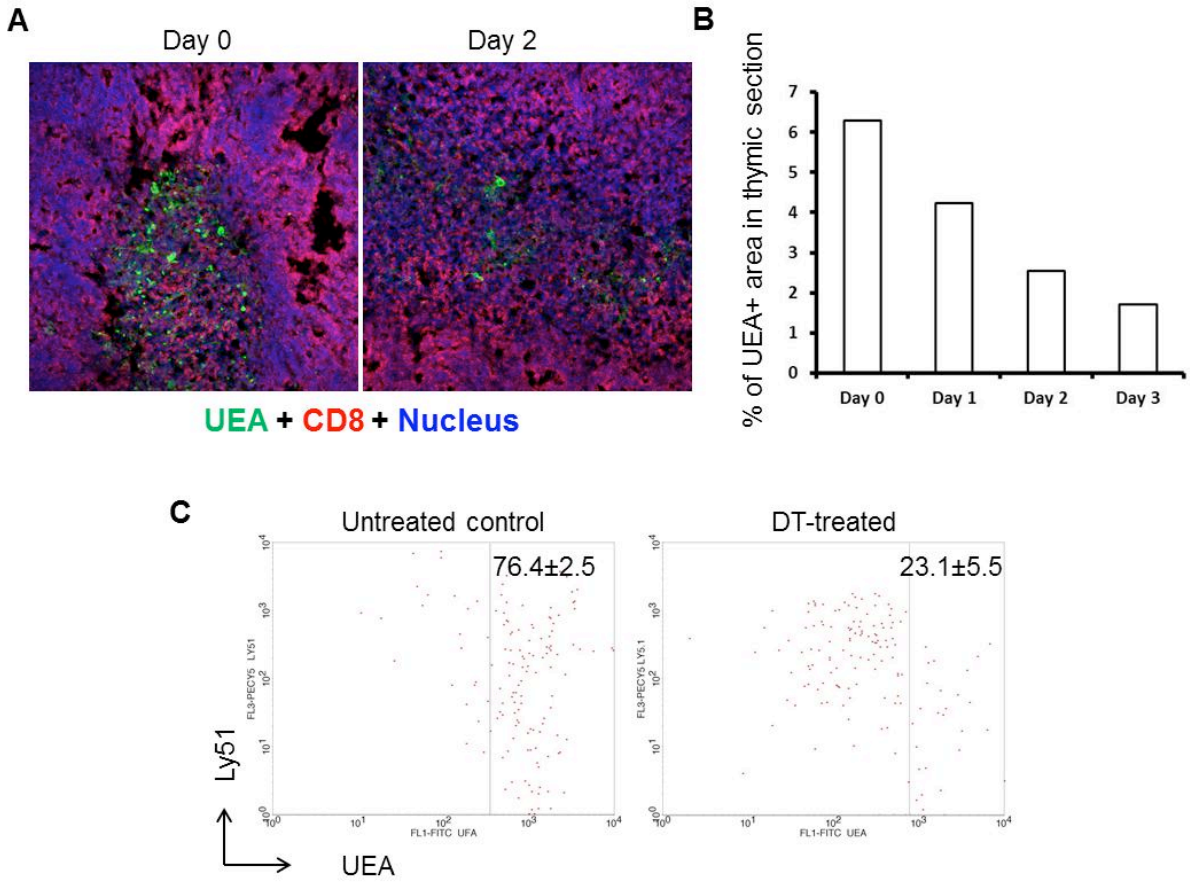

Figure 2. DT-mediated depletion of mTECs in Aire-DTR animal model. A. Immunohistochemical analysis of thymi harvested from DT-treated Aire-DTR mice. Thymic sections of 5um were stained with anti-CD8 antibody (red), in conjunction with UEA (green). Shown are representative images of untreated (day 0, left) and day2 (right) thymic sections. **B.** Gradual loss of mTECs in DT-treated thymi. Analysis of UEA+ mTEC areas was performed using Image J software. At least 5 separate sections from two animals were characterized at each time point. **C.** FCM analyses of thymic epithelial cells in control and DT-treated mice (5 days post DT-administration). Shown are representative FCM results from three independent experiments. Events are gated on thymic epithelial cells, including both cTECs and mTECs ($CD45^{-}g8.8^{+}$). Percentages of mTECs in the TEC population are shown.

Quarterly Summary:

In this quarter, we further quantified the numbers of insulin mRNA transcripts present in Aire-expressing splenocytes, and showed that insulin ectopic expression in Aire-expressing splenocytes is at a level about 1% of the house-keeping Hprt gene (a moderate expresser) and about 1/10^5 of islet cells. Our data also suggested that the primary role of insulin expression in hematopoietic lineage-derived cells is immunological relevant, as no abnormalities in hemacrit and metabolites were observed in animals in which hematopoietic insulin is abrogated. Last, we showed that our protocol of DT administration can effectively deplete mTECs in Aire-DTR animals, which allow us to investigate further the role of mTECs in regulating negative selection of self-reactive thymocytes.

Plans or milestones for the next quarter

We will examine thymocyte development in DT-treated Aire-DTR mice. Within the thymus, CD4+CD8+ DP thymocytes surviving the positive selection migrate from the cortical region into the medulla to further differentiate into SP thymocytes, where they will undergo negative selection. Since both thymic APCs and mTECs have been shown to mediate the negative selection process, study the impact of mTEC depletion will help us to understand mechanistically how autoreactive T cells are efficiently deleted in the thymus in order to establish a self-tolerant T cell repertoire.

Since self-antigen presentation has been implicated in natural T regulatory cell development, we will also examine the importance of mTECs in T regulatory cell generation in the thymus.

In the second quarterly scientific progress report (12/28/11 - 03/27/12) of year 03, we now report on our new and cumulative results.

Background and Significance:

Low levels of **t**issue **s**pecific **a**ntigens (TSA) mRNA transcripts have been found in both central and peripheral lymphoid organs, and have been implicated in establishing and maintaining the immunologic tolerance of peripheral organs and tissues. One of the key regulators of TSA ectopic expression is the autoimmune regulator Aire gene. Mice with Aire-deficiency develop clinical symptoms similar to human autoimmune polyendocrine syndrome type 1 (APS1): displaying autoimmune disorders in multiple organs. It was thus hypothesized that in situ TSA expression in thymic medulla is an essential and effective way for the adaptive immune system to acquire the molecular identities of non-circulating self-antigens in the periphery. Developing thymocytes with TCR specificity to TSAs are thus eliminated within the thymus through the central negative selection mechanism. Indeed, when insulin expression is specifically abrogated in medullary thymic epithelial cells (mTECs), animals develop autoimmune diabetes as early as three weeks after birth, due to insulin-specific autoreactive T-cell mediated rapid destruction of pancreatic b-cells. Although less is known about the immunologic function of TSA expression in peripheral lymphoid organs, we have demonstrated recently that splenic insulin expression is largely restricted to a population of Aire-expressing, $MHCII^{+}B220^{+}CD11c^{Int}$ antigen presenting cells (APCs) of hematopoietic origin, whereas ICA69 mRNA transcripts are predominantly present in CD45-Aire+ stromal cells in the lymph nodes.

It remains largely unknown how such low levels of TSA expression by a small number of stromal cells (~100,000 mTECs per mouse thymus) can negatively select billions of thymocytes effectively. Some of the key questions remained to be answered are: 1) whether the promiscuous levels of TSA expression in stromal cells of immune organs is the result of a large number of stromal cells with low mRNA transcripts, or reflect a small number of stromal cells expressing intermediate to high levels of TSAs; 2) whether stromal cells expressing the same TSAs are derived from the same progenitor cells (which will geometrically cluster together); 3) whether stromal cells derived from the same progenitors express the same TSA at the similar levels; 4) whether TSA expression in lymph node stroma follows the same rule as that of thymic medulla. Finding the answers to these questions will not only help us to understand mechanistically the biology of negative selection at cellular levels, but also facilitate our attempt to develop therapeutics to correct any genetic abnormalities in patients with autoimmune disorders like Type 1 diabetes.

Experimental Results

1. Multiplex in situ hybridization detection of insulin transcripts in pancreatic beta cells.

In this quarter, we took a novel approach to address the above questions: using the recently developed multiplex in situ hybridization technique, which enables us to detect multiple islet-specific autoantigen transcripts in a single cell of the stroma of either the primary or secondary lymphoid organs simultaneously. Since many factors, including proteinase treatment time, RNase contamination, probe-specificities et al., can all affect the final outcome of the results, in situ hybridization-based techniques are notoriously challenging. As a first step, we developed a set of 8 non-overlapping RNA probes targeting the mouse *Ins2* mRNA. To test the specificity of the probes and to optimize the experimental conditions for insulin transcripts detection in situ, we performed multiplex in situ hybridization on cryosections of pancreata (Figure 1). Without proteinase treatment, no insulin transcript signal was detectable (data not shown). Next, we subjected the pancreatic cryosections to 1 minute and 3 minutes of proteinase K treatment. As shown in Figure 1B and 1C, we were able to detect insulin transcripts in beta cells of pancreatic islets after 1 minute of treatment, whereas the signals were decreased significantly when we extended the proteinase K treatment to 3 minutes (Figure 1D). We also successfully counter-stained the nuclei with DAPI for better identification of individual cells (Figure 1E).

Taken together, we have successfully worked out the multiplex in situ hybridization protocol in our hands. We are in the process of further optimizing the conditions and exploring the possibility of using this technique on primary and secondary lymphoid organs.

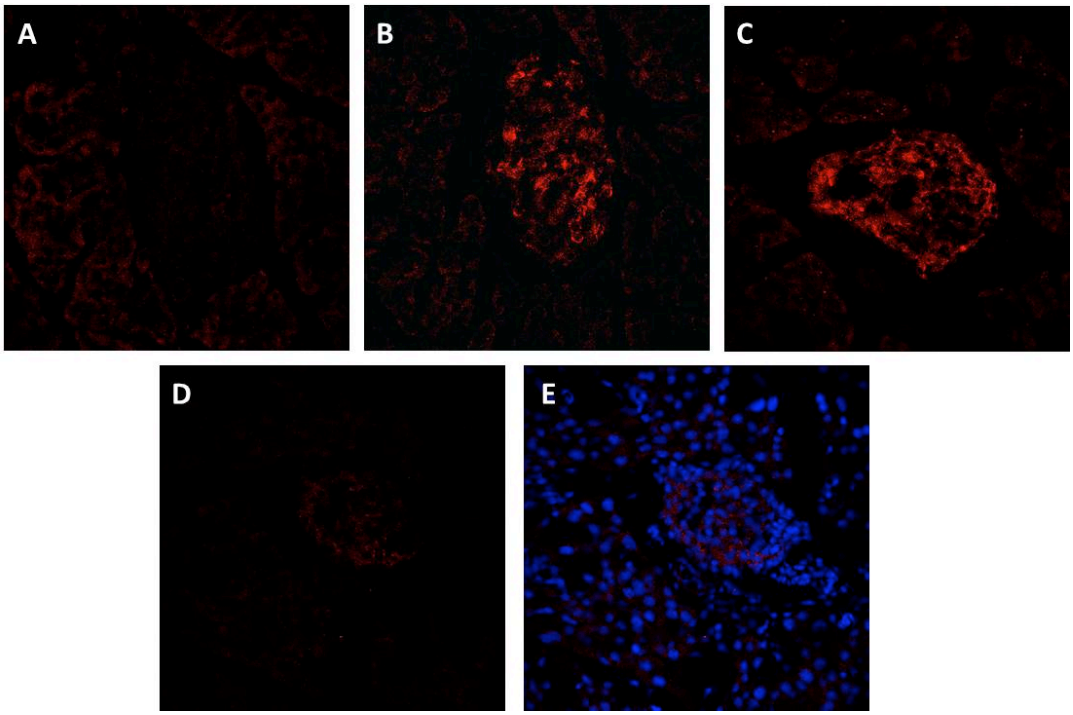

Figure 1. Detection of insulin transcripts in pancreatic beta cells with multiplex in situ hybridization. Pancreata were harvested from 8-week old mice and cryosectioned. **A-C**, proteinase K treatment for 1 minute. **D-E**, proteinase K treatment for 3 minutes. **A.** negative control without insulin RNA probe. **E.** Counter-stained with DAPI.

2. Detection of Aire-expressing cells in lamina propria.

As described previously, we have developed the Aire-Cre:iDTR mouse model (Aire-iDTR), in which human **d**iphtheria **t**oxin **r**ecepor (also called the heparin binding, EGF-like factor, HB-EGF) is specifically expressed in Aire-Cre expressing cells, rendering them sensitive to DT-induced apoptosis. As reported, we found that upon DT-administration, a gradual loss of mTECs was detected within the first three days. Two months post DT-treatment, Aire-iDTR mice develop autoimmunity in multiple organs, manifested as immune cell infiltration and expansion in liver, salivary glands, stomach, small intestines, large intestines and tear glands. However, the identities of Aire-expressing cells affected by DT-treatment were largely obtained from our characterization of the Aire-Cre: RosaYFP (Aire-iYFP) mice, in which YFP is presumably expressed on all the Aire-expressing cells. Whether the YFP expression pattern in the Aire-iYFP mouse can truthfully reflect the pattern of DT sensitive cells in the Aire-iDTR mouse and whether different populations of Aire-expressing cells are similarly sensitive to DT administrated (5ng/kg) remain unanswered. To further evaluate the impact of DT at the dose administrated, we developed, in this quarter, an immunohistochemistry-based method, which enabled us to examine directly the expression of DTR in Aire-expressing cells.

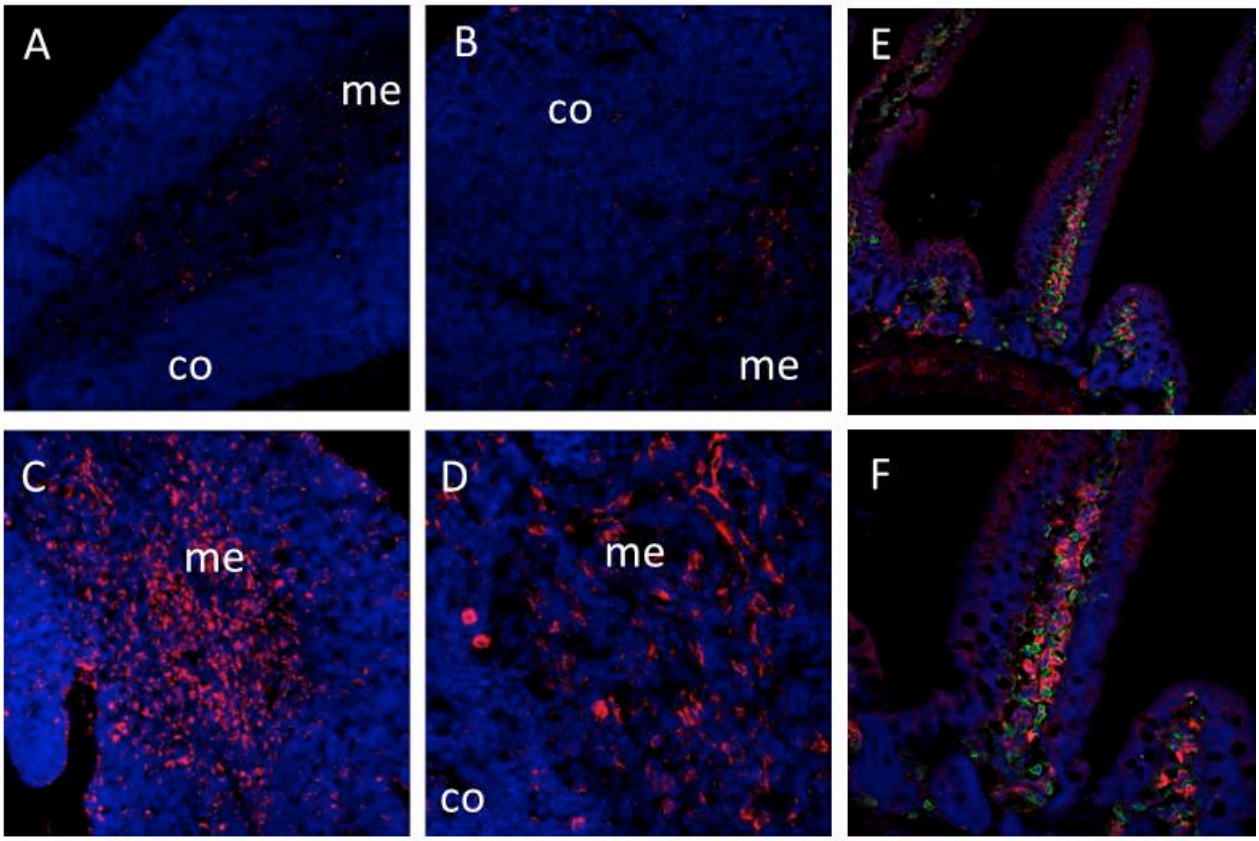

Figure 2. Immunohistochemical analysis of DTR expression. A-D, Cryosections of thymic samples harvested from negative control (**A** and **B**) and Aire-iDTR (**C** and **D**) mice were stained with a goat anti-DTR polyclonal antibody (red). **co**, cortical region; **me**, medullary region. **E** and **F**, DTR expression in lamina propria. DTR (red), CD45+ hematopoietic lineage cells (green). **A**, **C** and **E**, low magnification; **B**, **D** and **F**, high magnification.

As shown in Figure 2C and 2D, DTR expressing cells are largely restricted to medullary region of the thymus, consistent with the Aire expression pattern. In contrast, only background levels of DTR signals were detected in control thymus (Figure 2A and 2B), indicating that the anti-DTR antibody is not cross-reacting with the endogenous mouse HB-EGF proteins.

Of note, DTR-expressing cells were found also in lamina propria in the intestines, which were in close contact with CD45+ immune cells, suggesting a role of Aire-expressing cells in maintaining gut immune homeostasis. We are currently pursuing this hypothesis actively.

Future Directions and Milestones

In this quarter, we have developed a novel method, which will enable us to detect multiple islet autoantigen transcripts simultaneously. Furthermore, we developed an immunohistochemical protocol, allowing us to direct assess the impact of DT-administration in different populations of Aire-expressing cells. Finally, we found Aire-expressing cells in lamina propria, an important immune regulatory site for gut homeostasis.

In the next quarter, we will continue to optimize the multiplex in situ hybridization conditions and test it on primary immune organs. In addition, we will assess the DT-mediated depletion efficiency on different populations of Aire-expressing cells. Furthermore, the role of Aire-expressing cells in lamina propria will be examined.

In the third quarterly scientific progress report (03/28/12 - 06/27/12) of year 03, we now report on our new and cumulative results.

In the project, we have studied the essential roles of low levels of tissue specific antigen (TSA) expression in both primary and secondary lymphoid organs in establishing adaptive immune tolerance of peripheral tissues and organs and in prevention of autoimmune disorders, such as type 1 diabetes (T1D). Using genetic engineered mice, we generated a number of animal models, either to abrogate the expression of specific TSAs, such as insulin, in lymphoid organs, or to induce apoptosis of Aire-expressing cells with diphtheria toxin (DT) administration. From the study of these animal models, we demonstrated that while the TSA-expression in central lymphoid organs is essential for establishing adaptive immune tolerance to peripheral tissues/organs, their production in secondary lymphoid organs is important to maintain peripheral immune tolerance by inhibiting the proliferation and expansion of autoreactive lymphocytes. In the past quarter, we focused most of our effort on refining these results and preparing manuscripts for publication.

Experimental Results

3. Improving multiplex in situ hybridization protocol to detect insulin transcripts in INS1 cells.

Previously, we reported our initial attempt to detect insulin transcripts in pancreatic beta cells, using the recently developed multiplex in situ hybridization technique. The goal is to develop a method that has enough sensitivity to detect multiple islet-specific autoantigen transcripts in stromal cells of both primary and secondary lymphoid organs. We showed that we were able to detect insulin transcripts in beta cells of pancreatic islets after 1 minute of proteinase K treatment, whereas the signals were decreased significantly when we extended the treatment to 3 minutes. Nevertheless, the overall signal level is rather low. Since there are thousands of copies of insulin transcript in a single pancreatic beta-cell, the current sensitivity is clearly not high enough to detect 1-2 copies of insulin transcripts present in thymic stromal cells. One potential problem is the high RNase activities in the pancreas, which makes it rather difficult to perform any RNA work without perifusion of the whole organ with RNase inhibitors first. Thus, the low signal to noise ratio observed in pancreatic cyrosections did not necessarily reflect a low sensitivity of the technology, but rather suggest that the pancreas is not an ideal sample to be used for the optimization of the protocol.

To optimize the in situ hybridization protocol, we changed the samples to a pancreatic beta cell line, the INS1 cells. As shown in Figure 1B, our protocol can easily detect insulin transcripts in INS1 cells. We tested three proteinase K treatment conditions: 1, 2 and 3 minutes. Next, we subjected the pancreatic cryosections to 1 minute and 3 minutes of proteinase K treatment. Although the strongest insulin transcript signals were observed in samples undergoing proteinase K treatment for 3 minutes, morphologies of the INS1 cells were compromised, and the samples were smaller. Thus, 1-2 minutes of proteinase K treatment will be used for further studies.

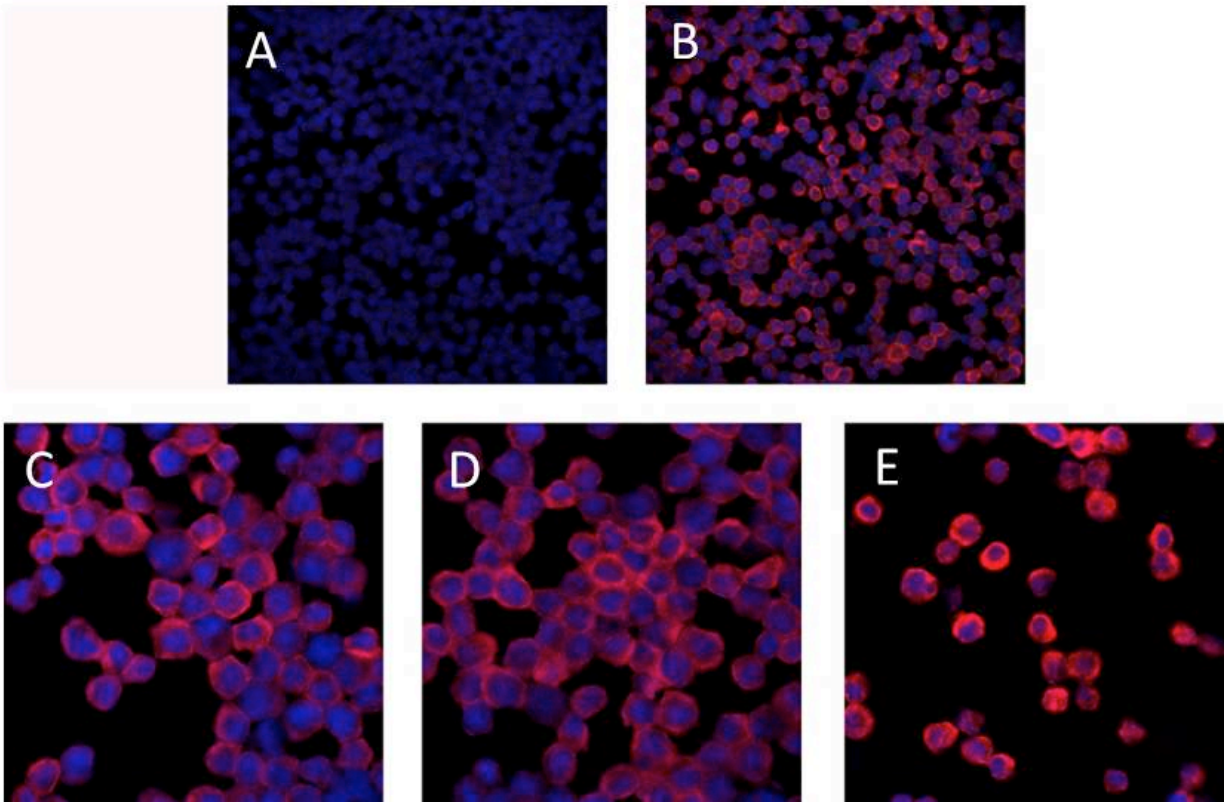

Figure 1. Detection of insulin transcripts in INS1 cells with multiplex in situ hybridization. INS1 cells were probed first with RNA probes complementary to insulin mRNA, followed by Alex 547-labeled hairpin RNA probes with self-assembling property (**B**, red). Nucleic DNA was counter-stained with DAPI (blue). **A**. Negative control without insulin-specific RNA probe. **C-E**, higher magnification microscopic images showing samples treated with 1, 2 and 3 minutes of proteinase K treatment, respectively.

Future Directions and Milestones

We are currently in the process of summarizing the data and refining our results for publication.

In the fourth and final quarterly scientific progress report (06/28/12 – 09/27/12) of Year 03, we now report on our cumulative findings for this entire project.

In this final report of the grant, we can summarize the key research accomplishments of the past three years.

Discrimination of self and nonself within the thymus environment is essential for the immune system to maintain a fine balance between tolerance and immunity. Defective central negative selection leads to the release of autoreactive T-cells into circulation, resulting in autoimmune responses to tissues and organs in the periphery. While the roles of thymic dendritic cells and macrophages in mediating tolerance of ubiquitously expressed self-molecules have long been established, only in the past decade, significant progress were made in understanding the mechanism of central tolerance to tissue specific antigens (TSAs), after the role of autoimmune regulator (Aire) gene in regulating low levels TSA expression in the thymic medullary epithelial cells (mTECs) was discovered. Mutations in Aire are the primary causes for human autoimmune polyglandular syndrome Type 1 (APS-1), a monogenic, multi-organ autoimmune disorder. It is now largely believed that Aire-controlled TSA expression in Aire+ mTECs provides ligands for negative selection of cognate T-cells, which is essential for the establishment of a self-tolerant T-cell repertoire.

However, not all thymic TSA transcription is driven by the Aire gene, although their thymic expression is as important as those Aire-regulated ones in providing ligands for negative selection of cognate T-cells. We have recently found that thymic expression of islet auto-antigen 69 (ICA69, encoded by the Ica1 gene) is not negatively regulated by Aire-deficiency, even if decrease of thymic Ica1 expression correlates inversely with anti-islet autoimmunity. Moreover, autoimmunity against alpha-fordrin was observed in Aire-deficient mice, despite that its thymic expression was largely unaffected. In addition, Aire knockout resulted in significant reduction of thymic Ins2 expression, but neither anti-insulin nor anti-islet autoimmunity was reported in Aire-deficient mice. Rather than the islet beta-cells, the exocrine acinar cells were the primary targets of autoimmune destruction in Aire-deficient NOD mice. Thus, the traditional view that mTECs mediate self-tolerance through Aire-regulating TSA expression is likely over-simplified; and additional Aire-dependent or independent tolerogenic mechanisms might exist in Aire-expressing mTECs (Aire+ mTECs) to protect from autoimmunity.

To establish a direct functional link between islet autoantigen expression in Aire+ mTECs and b-cell immune tolerance, we generated the ID-TEC animal model (for insulin-deletion in thymic epithelial cells), in which the mouse Ins2 gene is specifically depleted in Aire+ mTECs, whereas Ins2 expression in pancreatic b-cells remains intact. When crossed to Ins1-knockout background, ID-TEC mice develop type 1 diabetes spontaneously three weeks after birth. This was attributed to insulin-specific T-cell mediated pancreatic b-cells destruction. Results from our ID-TEC model and other groups have further demonstrated an indispensable role of islet autoantigen expression in Aire+ mTECs in establishing central immune tolerance to pancreatic b-cells and modulating T1D progression. Of note, the major histocompatibility complex (MHC) haplotype of ID-TEC mouse is H-2^b, which normally confers resistance to T1D development. Loss of thymic expression of islet autoantigens plays a dominant role for the survival of islet-specific autoreactive thymocytes through negative selection, over the protection of diabetes resistant MHC molecules. Thus, introducing T1D-resistant MHC molecules (e.g. H-2^b or H-2^d in mice) to progenitors of immune cells through genetic engineering might not be sufficient to eliminate islet-specific autoimmunity in susceptible individuals carrying T1D-prone MHC (e.g. H-2^{g7} in NOD mice).

Indeed, while recovery of pancreatic b-cell function, as manifested by increased, circulating C-peptide levels upon intraperitoneal glucose challenge, was observed in newly onset NOD mice that were non-myeloablatively conditioned and reconstructed with bone marrow progenitor cells transduced with IA^d-expressing retroviral particles, immune cells of donor origins were found to infiltrate the residual islets. These results suggest that while introducing T1D-resistant MHC molecules can alleviate the anti-islet autoimmune responses and delay T1D progression, the treatment is not sufficient to prevent the recurrence of islet autoimmunity.

To further improve our understanding of the immunomodulatory roles of islet TSAs in immune cells, we also developed the Aire-cre:RosaYFP animal models, which can effectively label all the Aire+ cells with

fluorescent marker EYFP. This approach not only enabled us to efficiently isolate the specific subsets of Aire+ mTECs from all the TECs in the thymus for further phenotypic and functional characterization, but it also allowed us to identify the population of islet TSA-expressing immune cells of tolerogenic nature in the periphery (Figure 1). The findings that there exist peripheral Aire+ tolerogenic antigen presenting cells (Aire+APCs) in both the spleen and the lymph nodes prompted us to investigate their potential roles in maintaining islet immune tolerance further.

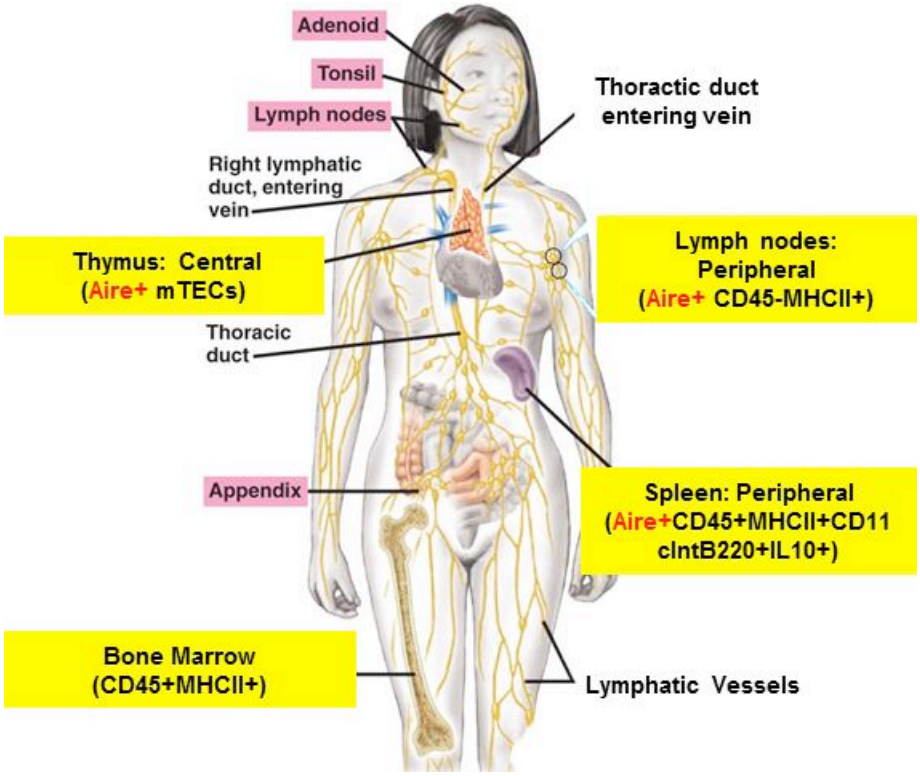

Figure 1. Aire-expressing cells in central and peripheral immune organs. Cells specified in the yellow squares are potential targets of genetic engineering to regain islet immune tolerance in T1D patients.

To investigate the roles of peripheral Aire+ APCs, we first performed phenotypic characterization of the Aire+ APCs in the periphery. As presented in the previous report, both CD45- (within the stroma of lymph nodes) and CD45+ (in the spleen) Aire+APCs express islet-specific autoantigens, such as insulin and ICA69, as well as other TSAs. In addition, both populations express MHCII molecules at high levels, an indicator of direct engagement with CD4+ T-cells. Furthermore, CD45+Aire+APCs in the spleen display many molecular features of tolerogenic APCs (B220+CD11cint), suggesting their roles in tolerance induction. Moreover, these cells produce high levels of tolerogenic cytoline, interleukin 10 (IL-10), implicating further a role in tolerance induction of pancreatic b-cells in T1D prone individuals.

To investigate the potential tolerogenic roles of Aire+APCs in T1D prevention, we took a genetic approach, generating animal models in which the insulin expression in either BM-derived or CD11c+ dendritic cells are specifically abolished. In the presence of diabetes-resistant MHC (e.g. H-2b haplotype), no insulitis or anti-islet autoimmunity was observed, indicating a dominant role of central immune organ, the thymus, in establishing immune tolerance of islet b-cells. However, when the genetic modification was moved to a background with faulty central negative selection (e.g. with H-2^{g7} haplotype), elevated levels of insulitis was observed, in comparison to B6. H-2^{g7} control mice. Our results demonstrate that Aire+ tolerogenic DCs play significant roles in mediating peripheral tolerance of islet b-cells. Significantly, our findings suggest that these peripheral Aire+APCs are the optimal targets for developing gene therapy based therapeutics to prevent and stop T1D.

KEY RESEARCH ACCOMPLISHMENTS:

Year 1:

- We have successfully demonstrated that BM-derived cells carrying the MHC class II IA^d molecules can confer resistance to type 1 diabetes.

- Development of transplantation protocols that can increase the efficacy of engraftment of the engineered hematopoietic precursor cells following non-radiation based preconditioning. A regimen with the combination of two antibodies (anti-CD52/CAMPATH and anti-cKit antibodies) was tested and proven to be successful for the repopulation of donor cells in the recipients.

- We have successfully generated the Aire-Cre:RosaYFP transgenic line, which enable us to identify two populations of insulin-expressing immune cells in the peripheral lymphoid organs: the $Aire^+CD45^+MHCII^+CD11c^{int}$ splenocytes of hematopoietic lineage and the $Aire^+CD45^-MHCII^+$ stromal cells in the lymph nodes.

Year 2:

- We generated the B6 H-2^b.ID-DC and B6 H-2^b.ID-BMC animal models in which insulin expression is abrogated specifically in bone marrow derived cells. These animals are free of diabetes, indicating further the dominant roles of central mechanism in T1D etiology.

- The roles of peripheral Aire-expressing cells in immune tolerance induction were investigated. We found direct contacts between Aire-expressing ($Aire^+$) cells and T regulatory (T_{reg}) cells in both the spleen and lymph node, suggesting a role of $Aire^+$ cells in supporting the survival and suppressive function of natural Treg cells. We also demonstrated that $Aire^+$ cells express high levels of immune tolerogenic cytokine, IL-10, suggesting its role in inducing the conversion of effector T-cells to inducible Tregs in the periphery.

- We have developed the Aire-iDTR animal model to further investigate the roles of mTECs in mediating islet immune tolerance. Administration of diphtheria toxin (DT) is sufficient to induce apoptosis of Aire-expressing mTECs.

Year 3:

4. We have successfully generated and characterized the B6.H-2^{g7}.ID-DC mice, in which insulin expression is specifically knocked out in both classic and tolerogenic dendritic cells and the animals carry the diabetes-prone MHC molecules. Although full-blown diabetes was not observed in these animals, they develop more severe insulitis, in comparison to B6. H-2^{g7} control mice. Our results not only demonstrate further the dominant role of central negative selection in establishing immune tolerance, but also indicate that Aire+ tolerogenic DCs play significant roles in mediating peripheral tolerance of islet b-cells.

5. We developed a protocol to identify $Foxp3^+$ T_{reg} cells in $EGFP^+$ T-cells without losing their EGFP marker, using flow cytometry technique. This technique will characterize different populations of cells with intracellular staining, while allowing us to identify the origin of cells at the same time.

6. Using the Aire-iDTR animal model, we show the dynamics of mTECs at different developing stage. Abrogation of $Aire^+$ mTECs can effectively abolish the expression and presentation of TSAs, resulting in multiple organ autoimmunity. Our data also demonstrate that after depletion of Aire+ mTECs, a population of $Aire^-$ mTECs can differentiate into Aire+ mTECs, and regain their TSA-expression capability.

REPORTABLE OUTCOMES:

1. Fan Y, Rudert WA, Grupillo M, He J, Sisino G and Trucco M (2009). Thymus-specific deletion of insulin induces autoimmune diabetes. The EMBO Journal 28: 2812-2824. (Feature article of the issue. Selected as Faculty of 1000 exceptional paper with F1000 factor 12.0.

2. Fan Y, Menon RK, Cohen P, Hwang D, Clemens T, DiGirolamo DJ, Kopchick JJ, LeRoith D, Trucco M, Sperling M (2009). Liver-specific deletion of the growth hormone receptor reveals essential role of GH signaling in hepatic lipid metabolism. Journal Biological Chemistry 284: 19937-19944. (Selected as paper of the week, ranked top 1% of all 6600 papers published on JBC annually).

3. Lu C, Kumar PA, Fan Y, Sperling MA, Menon R (2010) A novel effect of GH on macrophage modulates macrophage-dependent adipocyte differentiation. *Endocrinology* 151 (5): 2189-2199.

4. Perdomo G, Kim DH, Zhang T, Qu S, Thomas EA, Toledo FG, Slusher S, Fan Y, Kelley DE, Dong H (2010). A role of apolipoprotein D in triglyceride metabolism. *Journal of Lipid Research* 51: 1298-1311.

5. Mavalli M, DiGirolamo D, Fan Y, Riddle R, Campbell K, Sperling M, Frank S, Bamman M, Clemens T (2010). Distinct growth hormone receptor signaling modes control skeletal muscle development and insulin sensitivity. Manuscript accepted for publication in the *Journal of Clinical Investigation* 120 (11) 4007-20.

6. Trucco M (2010). Beta-cell regeneration: from science fiction to challenging reality. *Pediatr Diabetes* 11(5):292-5.

7. Trucco M (2010). Gene-environment interaction in type 1 diabetes mellitus. *Endocrinol Nutr.* 56 Suppl 4:56-9.

8. Kim DH, Perdomo G, Zhang T, Slusher S, Lee S, Phillips BE, Fan Y, Giannoukakis N, Gramignoli R, Strom S, Ringquist S, Dong HH (2011). Forkhead Box O6 Integrates Insulin Signaling With Gluconeogenesis in the Liver. Diabetes 60(11):2763-74.

9. Grupillo M, Lakomy R, Geng X, Styche A, Rudert WA, Trucco M, Fan Y (2011). An improved staining protocol for efficient detection of nuclear proteins in YFP-expressing cells. *Biotechniques* 51 (6): 417-420.

10. Grupillo M, Gualtierotti G, He J, Sisino G, Bottino R, Rudert WA, Trucco M, Fan Y (2012). Essential roles of insulin expression in Aire+ tolerogenic dendritic cells in maintaining peripheral self-tolerance of islet ß-cells. *Cellular Immunology* 257(2): 115-123.

11. Bonner SM, Pietropaolo SL, Fan Y, Chang Y, Sethupathy P, Morran MP, Beems M, Giannoukakis N, Trucco G, Palumbo MO, Solimena M, Pugliese A, Polychronakos C, Trucco M, Pietropaolo M (2012). Sequence Variation In the Promoter of the Ica1 gene, which encodes a protein implicated in type 1 diabetes, causes the Transcription Factor AIRE to Increase Its Binding and downregulate Expression. *Journal of Biological Chemistry* 287: 17882-17893.

CONCLUSIONS

The work performed during this study illustrated the pivotal roles of islet-autoantigen expressing immune cells in the stroma of both central and peripheral lymphoid organs in establishing and refining immune tolerance of islet b cells. Our work demonstrates the dominant roles of central negative selection in establishing immune tolerance, and also indicates a pivotal role of $Aire^{+}$ tolerogenic antigen presenting cells in maintaining peripheral tolerance of islet b-cells. Thus, a successful gene therapy regimen will take both arms into consideration to guarantee its success.

Based upon these results, we propose to increase the islet-autoantigen expression in thymic medullary epithelial cells through genetic engineering as a therapeutic to treat T1D patients. This approach will prevent the nascent generation of islet targeting autoreactive lymphocytes within the thymus. In addition, circulating $Aire^{+}$ tolerogenic APCs can be enriched and engineered to express elevated levels of islet-autoantigens, which will presumably prevent the expansion islet-reactive lymphocytes.

BIBLIOGRAPHY OF PUBLICATIONS

1. Fan Y, Rudert WA, Grupillo M, He J, Sisino G and Trucco M (2009). Thymus-specific deletion of insulin induces autoimmune diabetes. The EMBO Journal 28: 2812-2824. (Feature article of the issue. Selected as Faculty of 1000 exceptional paper with F1000 factor 12.0.

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11. Bonner SM, Pietropaolo SL, Fan Y, Chang Y, Sethupathy P, Morran MP, Beems M, Giannoukakis N, Trucco G, Palumbo MO, Solimena M, Pugliese A, Polychronakos C, Trucco M, Pietropaolo M (2012). Sequence Variation In the Promoter of the Ica1 gene, which encodes a protein implicated in type 1 diabetes, causes the Transcription Factor AIRE to Increase Its Binding and downregulate Expression. *Journal of Biological Chemistry* 287: 17882-17893.

Highlighted publications are included with this report.

Personnel receiving pay from the research effort, W81XWH-09-1-0742

2009 – 2010

Suzanne Bertera, Ph.D.
Yong Fan, Ph.D.
Xuehui Geng, Ph.D.
Jing He, M.D., Ph.D.
Bernice Johns
Darleen Noah
Massimo Trucco, M.D.
Tatyana Votyakova, Ph.D.
Jennifer Zeak

2010 – 2011

Suzanne Bertera, Ph.D.
Rita Bottino, Ph.D.
Yong Fan, Ph.D.
Joseph Flounlacker
Amber Funair
Xuehui Geng, Ph.D.
Jing He, M.D., Ph.D.
Bernice Johns
Carmella Knoll
Michael Knoll
Robert Lakomy
Darleen Noah
Ann Piccirillo
William Rudert, M.D., Ph.D.
Theodore Scheide
Alexis Styche
Massimo Trucco, M.D.
Tatyana Votyakova, Ph.D.
Catarina Wong

2011 – 2012

Yong Fan, Ph.D.
Massimo Trucco, M.D.

The EMBO Journal (2009) 28, 2812–2824 | © 2009 European Molecular Biology Organization | All Rights Reserved 0261-4189/09
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THE EMBO JOURNAL

Thymus-specific deletion of insulin induces autoimmune diabetes

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Insulin expression in the thymus has been implicated in regulating the negative selection of autoreactive T cells and in mediating the central immune tolerance towards pancreatic β-cells. To further explore the function of this ectopic insulin expression, we knocked out the mouse *Ins2* gene specifically in the *Aire*-expressing medullary thymic epithelial cells (mTECs), without affecting its expression in the β-cells. When further crossed to the *Ins1* knockout background, both male and female pups (designated as ID-TEC mice for insulin-deleted mTEC) developed diabetes spontaneously around 3 weeks after birth. β-cell-specific autoimmune destruction was observed, as well as islet-specific T cell infiltration. The presence of insulin-specific effector T cells was shown using ELISPOT assays and adoptive T cell transfer experiments. Results from thymus transplantation experiments proved further that depletion of *Ins2* expression in mTECs was sufficient to break central tolerance and induce anti-insulin autoimmunity. Our observations may explain the rare cases of type 1 diabetes onset in very young children carrying diabetes-resistant HLA class II alleles. ID-TEC mice could serve as a new model for studying this pathology.

The EMBO Journal (2009) **28,** 2812–2824. doi:10.1038/emboj.2009.212; Published online 13 August 2009
Subject Categories: immunology
Keywords: autoantigen; central tolerance; insulin; thymic medullary epithelial cells; type 1 diabetes

Introduction

As a multi-factorial, multigenic metabolic disorder, type 1 diabetes (T1D) is promoted by chronic autoimmune destruction of the insulin-producing pancreatic β-cells, whereas other endocrine cells within the islets and the surrounding exocrine cells are largely spared (Tisch and McDevitt, 1996). Such cellular specificity strongly implicates the fundamental role of β-cell-specific self-antigens in the initiation and progression of the disease. It is well established that specific major histocompatibility complex (MHC) alleles are major contributors of genetic susceptibility to T1D (Todd *et al*, 1987; Morel *et al*, 1988). Autoreactive T cells are released into the circulation because of the faulty presentation of self-antigens by disease-susceptible MHC molecules, which hinder the negative selection process in the thymus (Trucco, 1992; McDevitt, 2001). To date, only two antigens solely expressed in β-cells have been identified: insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Lieberman *et al*, 2003; Gianani and Eisenbarth, 2005). Cytotoxic T cells specific for either antigen were isolated from naïve NOD mice, and were able to transfer T1D to NOD–*scid* mice. However, insulin may have a primary function, because the immune response against IGRP could be prevented by inducing tolerance to (pro)insulin in NOD mice (Krishnamurthy *et al*, 2006). Indeed, in pre-diabetic patients, insulin-specific autoantibodies are detected frequently, long before the onset of clinical symptoms, and their affinities correlate with the progression of the autoimmune attack (Achenbach *et al*, 2004). Similarly, insulin-specific T cells are predominant components of the islet-infiltrating T cells at the pre-diabetic stage in NOD mice. Multiple T cell clones, targeting different insulin epitopes, have been isolated, including both $CD4^+$ and $CD8^+$ T lymphocyte sub-types (Wegmann *et al*, 1994). Unlike other known autoantigens identified in T1D, such as glutamic acid decarboxylase 65 (GAD 65), tyrosine phosphatase-like protein, IA-2 and IGRP, (pro)insulin is the only autoantigen for which transgenic overexpression in antigen presenting cells (APCs) affects the disease progression in NOD mice (Gianani and Eisenbarth, 2005). Moreover, when a metabolically active, mutant form of insulin (which has alanine in place of tyrosine at the 16th amino-acid position of the B-chain) was transgenically expressed in insulin knockout NOD mice, insulitis and the development of autoimmune diabetes were totally abolished (Nakayama *et al*, 2005). The altered sequence presumably changes the antigenicity of the dominant insulin B9–23 peptide. These findings further implicate the function of insulin in the autoimmune etiopathogenesis of T1D.

The broad range of tissue-specific genes that are ectopically expressed in the thymus, and the seminal studies of *Aire* knockouts highlight the importance of central selection mechanisms in establishing and maintaining a T cell repertoire that is tolerant towards peripheral tissues (Anderson *et al*, 2002; Kyewski and Klein, 2006). As a putative transcription factor, *Aire* was shown to promote the thymic expression of a number of peripheral tissue antigens (PTAs), such as *Mucin-6* (stomach specific) and interphotoreceptor retinoid-binding protein (*Irbp*, eye specific), which facilitate self/non-self distinction (DeVoss *et al*, 2006; Gavanescu *et al*, 2007). The presentation of PTAs in the thymus enabled the deletion of high-affinity, potentially autoreactive T cells through negative selection. In the *Aire* knockouts, massive T cell infiltration in multiple solid organs was observed following downregulation of *PTA* expression in the thymus (Anderson *et al*, 2005).

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Received: 25 March 2009; accepted: 1 July 2009; published online: 13 August 2009

Indeed, when the thymus of an *Irbp*-knockout mouse was transplanted under the kidney capsule of the thymus atrophic nude mouse, T cell infiltration was detected in the eyes after 10 weeks, suggesting that failure of thymic expression of peripheral antigens is sufficient to cause organ-specific autoimmunity (DeVoss *et al*, 2006).

Similar to other *Aire*-regulated PTAs, insulin expression has been found in the thymus (Jolicoeur *et al*, 1994). Variations of this expression may be especially relevant to diabetes. The human IDDM2 locus was mapped to a variable number tandem repeat (VNTR) region 5′ proximal to the insulin gene (Pugliese *et al*, 1997; Vafiadis *et al*, 1997). Different VNTR alleles correlate with different thymic insulin expression levels; alleles with shorter repeats result in a reduction of expression and predisposition to T1D. Such a correlation between levels of thymic insulin production and anti-insulin autoimmunity was also suggested by animal studies (Chentoufi and Polychronakos, 2002).

It remains controversial which cells in the thymus are expressing insulin. APCs of bone marrow origin, including thymic dendritic cells and macrophages were reported to express (pro)insulin transcripts (Pugliese *et al*, 2001). In contrast, studies using purified thymic cell populations showed that (pro)insulin was expressed only in thymic epithelial cells (TECs) of the endoderm origin (Palumbo *et al*, 2006). In an attempt to resolve this controversy, Faideau *et al* (2006) created bone marrow chimeras in *Ins2* knockout mice and showed that tolerance to (pro)insulin-2 was due to radioresistant cells in the thymus, presumably epithelial cells.

To address the importance of thymic insulin expression in mediating immune tolerance towards pancreatic β-cells even in the context of MHC allelic resistance (Pietropaolo *et al*, 2002), we first examined the *Ins2* ectopic expression in specific thymic cell types, and identified endoderm-derived medullary TECs (mTECs) as the major *Ins2*-expressing cells in the thymus, consistent with previous publications (Anderson *et al*, 2002; Palumbo *et al*, 2006). To elucidate the essential role of this mTEC-insulin expression, we developed an animal model in which the mouse *Ins2* gene was specifically deleted in mTECs, whereas its production in the pancreas remained intact. When these mTEC-*Ins2*-deleted animals also lacked (pro)insulin 1, the other isoform of the mouse insulin gene, spontaneous T1D developed at around 3 weeks after birth.

Our study showed that disruption of thymic expression of a single tissue-specific self-molecule is sufficient to trigger autoimmunity towards the relevant tissue and results in pathologic damage even in the presence of disease-resistant alleles of MHC molecules.

Results

Ins2 expression in medullary epithelial cells of the thymus

We chose the *Ins2* gene as the target in our study because it is the predominant isoform of mouse insulin expressed in the thymus (Chentoufi and Polychronakos, 2002), and because the *Ins2* gene alone is sufficient to maintain glucose homoeostasis in animals with the *Ins1* gene knocked out (Duvillié *et al*, 1997). To examine *Ins2* expression in specific cell types in the thymus of *Ins1* knockout mice, we used a magnetic bead-based isolation method in conjunction with flow cytometry to separate thymic stromal cells into four groups, based on their characteristic surface antigens and molecular signatures. Specifically, hematopoietic lineage-derived thymic APCs were separated as CD45$^+$, MHC class II$^+$, CD11c$^+$ thymic dendritic cells (R1) and CD45$^+$, MHC classII$^+$, CD11c$^-$ macrophages and B-cells (R2); whereas TECs were collected as CD45$^-$, EpCAM$^+$, Ly51^{low}, UEA^{high} medullary TECs (R3) and CD45$^-$, EpCAM$^+$, Ly51^{high}, UEA^{low} cortical TECs (R4) (Figure 1A). The purity of the separation was validated by RT–PCR analysis of the expression of cell type-specific genes: CD45 transcripts were only detectable in bone marrow-derived cells (R1 and R2), whereas cytokeratin 2/8 (K-2/8) transcripts were predominantly present in epithelial cells (R3 and R4). As shown in Figure 1B, and consistent with previous publications, mTECs are responsible for nearly all the ectopically expressed *Ins2* in the thymus (Anderson *et al*, 2002; Faideau *et al*, 2006; Palumbo *et al*, 2006).

Generation of an animal model to knockout Ins2 in Aire-expressing mTECs

To elucidate the physiological role of mTEC-specific *Ins2* expression, we chose to use the Cre–Lox system to specifically deplete insulin expression in these particular thymic cells, without affecting its production in pancreatic β-cells (see Supplementary Figure S1 for our strategy). As the first step, two *loxp* sites were genetically engineered to flank the

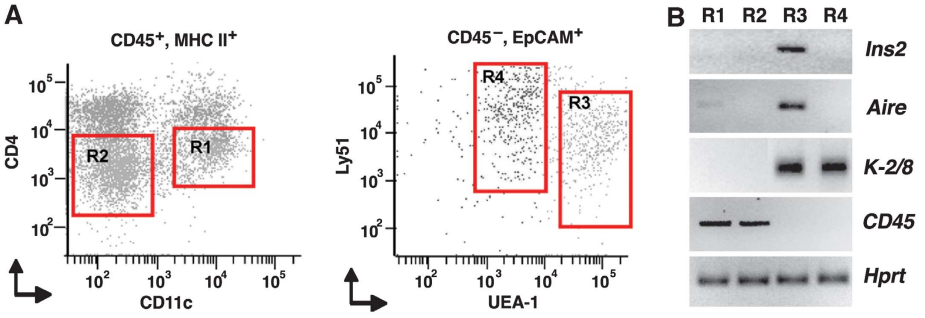

Figure 1 Analysis of *Ins2* expression in specific thymic stromal cells of *Ins1* knockout mice. (**A**) Isolation of thymic stromal cells. Left panel, fluorescence-activated cell sorting of bone marrow-derived antigen presenting cells. CD45$^+$, MHC II$^+$ cells were further gated for CD4$^-$, CD11c$^+$ thymic dendritic cells (R1), and CD4$^-$,CD11c$^-$ macrophages and B cells (R2). Right panel, CD45$^-$, EpCAM$^+$ stromal cells were sorted for medullary epithelial cells (R3: Ly51^{low}, UEA^{high}) and cortical epithelial cells (R4: Ly51^{high}, UEA^{low}). The same R1–R4 designation has been used throughout the paper unless otherwise specified. (**B**) RT–PCR analysis of the mouse *Ins2* gene expression in specific thymic antigen presenting cells (R1–R4). The house keeping *Hprt* gene expression was used as control. K-2/8, cytokeratin 2/8.

mouse *Ins2* gene (designated as $Ins2^{l}$) (Figure 2). Once homozygous $Ins2^{l/l}$ mice were obtained, they were crossed to *Ins1* knockout mice ($Ins1^{-/-}$) to generate double homozygous mice ($Ins1^{-/-}$:$Ins2^{l/l}$). These mice are viable and euglycemic throughout their lifespan, indicating that the inserted *loxp* sites did not affect insulin production and carbohydrate metabolism. Efficient deletion of the *Ins2* gene was validated by Southern blot analysis of DNA samples isolated from the offspring of $Ins2^{l/l}$ mice crossed to germline-expressing, EIIa-Cre mice (data not shown). Furthermore, when these germline-*Ins2*-deleted offspring were further crossed to $Ins1^{-/-}$ mice, double homozygous mice ($Ins1^{-/-}$:$Ins2^{-/-}$) died within 2–3 days postnatally, due to the absence of insulin in pancreatic β-cells (Supplementary Figure S2), in accordance with previous reports (Duvillié *et al*, 1997).

It has been shown that the *Aire* gene is expressed predominantly in cells of the thymic medullary epithelium (Figure 1B) and is able to regulate the ectopic expression of the *Ins2* gene in the thymus (Anderson *et al*, 2002). To target mTECs specifically, the putative transcription regulatory element, cloned from the murine *Aire* gene, was used to create an *Aire–Cre* transgene (Figure 3A). Two transgenic lines carrying multiple copies were established and one was selected for further study, as both showed similar expression patterns.

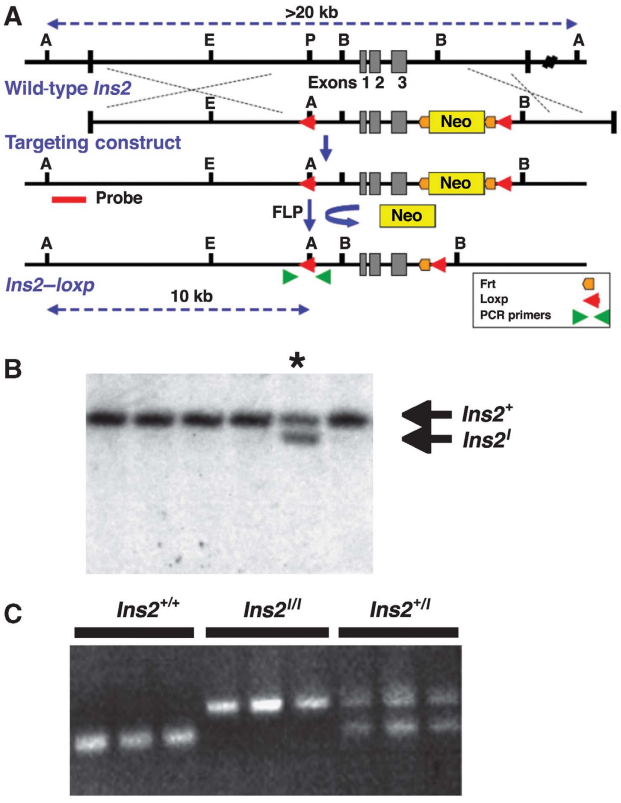

Figure 2 Genetic modification of the mouse *Ins2* gene for thymus-specific deletion. (**A**) A targeting construct with two *loxp* sites flanking the mouse *Ins2* gene and an *frt*-tagged *Neo* cassette for selection was used to modify the *Ins2* gene through homologous recombination (black dash line). *Neo* cassette within the *loxp*-tagged *Ins2* allele was deleted from the genome by FLP recombinase. A *Ase*I, B: *Bam*HI, E: *Eco*RI, red bar: 5′ external probe. (**B**) Southern blot analysis of *Ins2*-targeted embryonic stem cell clones. Genomic DNA was isolated, digested using *Ase*I and probed with the 5′ external probe. * indicates a *loxp*-tagged *Ins2* embryonic stem cell clone. As the result of the genetic modifications, an *Ase*I site was introduced to the *loxp*-tagged *Ins2* allele ($Ins2^{l}$), which appears as the extra 10-kb band on the blot, in addition to the 20-kb band from the wild-type allele ($Ins2^{+}$). (**C**) Agarose gel showing PCR-based genotyping of mice. +, wild type; *l*, *loxp*-tagged *Ins2* ($Ins2^{l}$).

To examine the expression pattern of the *Aire–Cre* transgene, we carried out RT–PCR to analyse the presence of *Cre* transcripts in a number of tissues, including the thymus (Figure 3A). As shown, the *Aire–Cre* transgene was predominantly expressed in the thymus, albeit very weak Cre signals were detected in other organs, such as the spleen, the kidney and the pancreas. To further characterize the *Aire–Cre* transgene expression within the thymus, thymic stromal cells were collected from *Aire–Cre* mice and were separated into the four groups (R1–R4) as described above (Figure 1A) and analysed by RT–PCR. As shown in Figure 3B, *Cre* transcripts were present only in the mTECs (R3), but not in the cortical TECs (R4), nor in cells of the hematopoietic lineages (R1 and R2), which is consistent with the expression pattern of the endogenous *Aire* gene. We also examined the presence of *Cre* transcripts 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* transcripts were observed (Figure 3B).

The effectiveness of the *Aire–Cre* transgene to delete *loxp*-tagged targeted genes was tested by crossing the Cre transgenic line to Rosa26R-lacZ reporter mice (Soriano 1999). In these animals, Cre-mediated recombination generates a functional *LacZ* gene, which is transcribed by means of the universally active Rosa26 promoter. The LacZ signal was only observed in the $CD45^{-}$ cells of the thymi of Aire–Cre:Rosa26R-lacZ mice, indicating the epithelial nature of the Cre expressing cells (Figure 3C). To rule out any Cre recombinase activity in the pancreatic β-cells in the transgenic lines, immunohistochemical analysis of the pancreata was carried out. Pancreatic *LacZ* expression was only detectable in control Rip–Cre:Rosa26R-lacZ mice, in which the expression of *Cre* transcripts was driven by the rat insulin promoter (Figure 3D). Thus, the use of the *Aire–Cre* transgene enabled us to target the floxed genes in specific cell types in the thymus.

To prove directly that the *Aire–Cre* transgene can delete the floxed *Ins2* gene in mTECs efficiently, we collected thymi from 6–8-week-old Aire–Cre:$Ins1^{-/+}$:$Ins2^{1/1}$ mice. Furthermore, thymic stromal cells were separated into the four groups (R1–R4). Genomic DNA isolated from each group was analysed by PCR using the primer pairs shown in Figure 2A. As the complementary sequence of the reverse primer was absent when the floxed *Ins2* gene was deleted from the genome, only the intact *Ins2* floxed allele could be amplified. As shown in Figure 3E, the PCR signal of the floxed *Ins2* gene is significantly lower in mTEC samples (R3) than those of other thymic stromal cells, indicating efficient deletion of the floxed *Ins2* gene in mTECs. To ensure that the *Aire–Cre* transgene would not cause deletion of the floxed *Ins2* gene in the pancreatic β-cells, islets were also isolated from these mice, and the genomic DNA was examined. The PCR signal of the floxed *Ins2* alleles was unaffected, consistent with results from the Rosa26R-lacZ reporter mice experiments (Figure 3E).

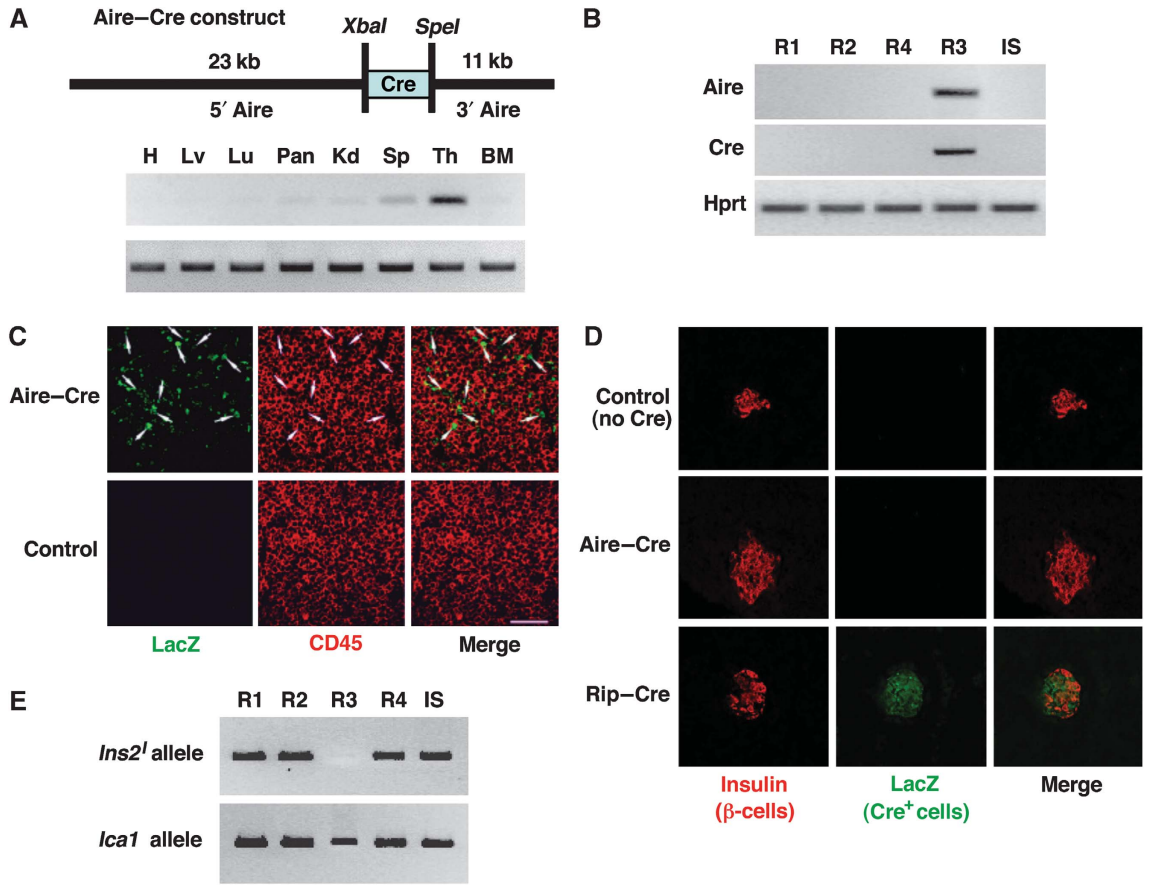

Figure 3 Characterization of *Aire–Cre* transgene expression. (**A**) Upper panel, schematic drawing of the *Aire–Cre* construct. A 23-kb and an 11-kb genomic fragment were subcloned from the 5′ and 3′ of mouse *Aire* gene, respectively, and engineered to regulate the Cre gene expression. Lower panel, RT–PCR analysis of *Aire–Cre* transgene expression. (**B**) RT–PCR analysis of Cre expression in specific thymic stromal cells (R1–R4) isolated from the *Aire–Cre* transgenic mice. (**C**) Aire–Cre:Rosa26R-lacZ thymus stained with anti-LacZ antibody (green) and CD45 antibody (red). LacZ$^+$ and CD45$^+$ cells (yellow). Arrows indicate LacZ$^+$, CD45$^-$ thymic epithelial cells. (**D**) Absence of Cre recombinase expression in pancreatic β-cells of *Aire–Cre* mice. Pancreas samples from six-week-old Aire–Cre:Rosa26R-lacZ, Rosa26R-lacZ (negative control), and Rip–Cre:Rosa26R-lacZ (rat insulin promoter, positive control) mice stained with anti-insulin (red) and anti-LacZ (green) antibodies. (**E**) Efficient deletion of the floxed *Ins2* allele in mTEC cells collected from Aire–Cre:*Ins1*$^{-/+}$:*Ins2*$^{l/l}$ mice. Genomic DNA was isolated from different thymic stromal cells (R1–R4) and pancreatic islets and the presence of the floxed *Ins2* allele was analysed using PCR, with the same primer pairs depicted in Figure 2B. PCR amplification of the mouse *Ica1* gene was used as control of the genomic DNA input. BM, bone marrow; H, heart; IS, islets; Kd, kidney; Lu, lung; Lv, liver; Pan, pancreas; Sp, spleen; Th, thymus.

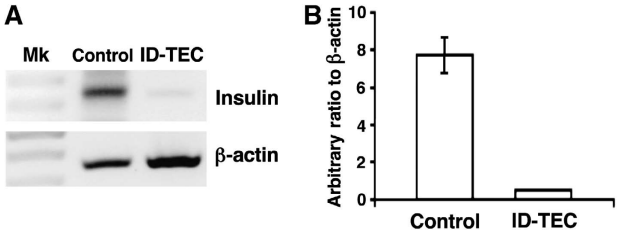

Figure 4 Thymic insulin expression in ID-TEC mice. (**A**) RT–PCR analysis of thymic insulin expression in ID-TEC mice. (**B**) Real-time PCR quantification of thymic insulin expression in ID-TEC and control mice. (Error bar: s.e.m.) Mk, molecular size marker.

Ecotopic thymic insulin expression in ID-TEC mice

On the basis of the above results, we proceeded to breed the *Aire–Cre* transgenic line with *Ins1*$^{-/-}$:*Ins2*$^{l/l}$ mice to homozygosity, generating the ID-TEC mice (insulin-deleted thymic epithelial cell; genotype: Aire–Cre:*Ins1*$^{-/-}$:*Ins2*$^{l/l}$), to knock out insulin expression specifically in the endoderm-derived thymic epithelial cells. As expected, according to Mendel's law, about a quarter of the newborns from the *Ins1*$^{-/-}$:*Ins2*$^{l/l}$ and Aire–Cre:*Ins1*$^{-/-}$:*Ins2*$^{+/l}$ mating were ID-TEC pups. Littermates with *Ins1*$^{-/-}$:*Ins2*$^{l/l}$, *Ins1*$^{-/-}$:*Ins2*$^{+/l}$, or Aire–Cre:*Ins1*$^{-/-}$:*Ins2*$^{+/l}$ genotypes were used as controls unless otherwise specified. The ID-TEC pups were born with normal size and body weight, indicating that the genetic manipulation did not affect their prenatal growth.

As predicted, the levels of thymic *Ins2* expression in ID-TEC mice were drastically lower than those of controls (*Ins1*$^{-/-}$:*Ins2*$^{l/l}$ mice) (Figure 4A). Real-time PCR analysis showed that thymic *Ins2* mRNA expression is knocked down to less than 10% of the controls (Figure 4B). The observed, very low residual insulin mRNA expression could come from the small population of bone marrow-derived, CD45$^+$ thymic APCs that may express low levels of insulin mRNA, or could be due to the reported abnormal upregulation of (pro)insulin expression in bone marrow-derived APCs under various experimental and/or hyperglycemic situations (Kojima *et al*, 2004; Carlsen and Cilio, 2008).

ID-TEC mice develop spontaneous diabetes within 3 weeks after birth

In early postnatal life, ID-TEC pups have normal size and weight compared with their control littermates and have normal blood sugar levels. When pancreata collected from newborn pups (day 1 postnatal) were examined by immunohistochemistry, islet structures were observed to be normal with abundant insulin-producing β-cells (Figure 5A). These data further confirmed the absence of *Aire–Cre* transgene expression in the islet β-cells of ID-TEC mice.

At postnatal day 10, ID-TEC mice remained normoglycemic with similar plasma insulin levels and pancreatic insulin contents compared with controls (Figure 5B and C). However, within the following week, ID-TEC pups started to develop elevated blood sugar levels (Figure 5D). By 3 weeks, in striking contrast to control mice, severe hyperglycemia developed in both male and female ID-TEC mice (Figure 5D). Consistently, circulating insulin levels were significantly lower than those of controls, indicating insufficient β-cell function (Figure 5E). Immunohistochemical examination of pancreata, collected from diabetic ID-TEC mice (blood glucose ~500–600 mg/100 ml), showed that only a small number of insulin-positive β-cells were still present at 4 weeks after birth, whereas glucagon-secreting α-cells remained largely intact (Figures 5F). In some islet-like areas, granulocytic invasion was prominent, indicating the active scavenging of islet tissues after acute damage (Supplementary Figure S3). Taken together, these data clearly showed that a drastic loss of insulin-producing β-cells occurred in ID-TEC mice during the postnatal time window of 2–3 weeks.

Insulin-specific, T cell mediated islet β-cell destruction in ID-TEC mice

The specific loss and damage of β-cells in ID-TEC mice lead us to investigate the autoimmune nature of the disease. As expected, pancreata collected from non-diabetic littermate controls were largely free of infiltrating lymphoctyes (Supplementary Figure S4), whereas numerous $CD4^+$ and $CD8^+$ T cells (i.e. insulitis) were found in diabetic ID-TEC pancreata when examined by immunohistochemistry at 3 weeks (Figure 6A–D). In addition, B-cells and macrophages are also observed in the islets, reflecting an ongoing inflammatory response (Figure 6E and F). Moreover, islet infiltrations of different severities were observed in pancreata of prediabetic 14-day-old ID-TEC pups (Supplementary Figure S5). To further characterize the anti-islet autoimmunity, we analysed the presence of anti-insulin autoantibody (IAA) in the sera of ID-TEC mice with an ELISA-based colorimetric assay. As shown in Figure 6G, the light absorption signals from ID-TEC sera were well above those from littermate controls, showing the existence of IAA in the circulation of ID-TEC mice (Figure 6H). Consistently, sera collected from 6–8-week-old diabetic ID-TEC mice were able to recognize islet cells in sections prepared from C57BL/6 $Rag1^{-/-}$ (*Rag1*) mice.

Interestingly, these clear signs of autoimmunity were found in the ID-TEC mice that, in fact, were derived from three mouse lines with either C57BL/6 (the Aire–Cre and $Ins1^{-/-}$ lines) or 129:C57BL/6 mixed (the $Ins2^{l/l}$ line) backgrounds, all of which carried the H-2^b MHC haplotype, which would normally conveys resistance to islet autoimmunity.

To examine the antigen-specificity of the T cells present in the ID-TEC mice, ELISPOT assays were carried out. On stimulation with insulin, IFN-γ-secreting T cells were found

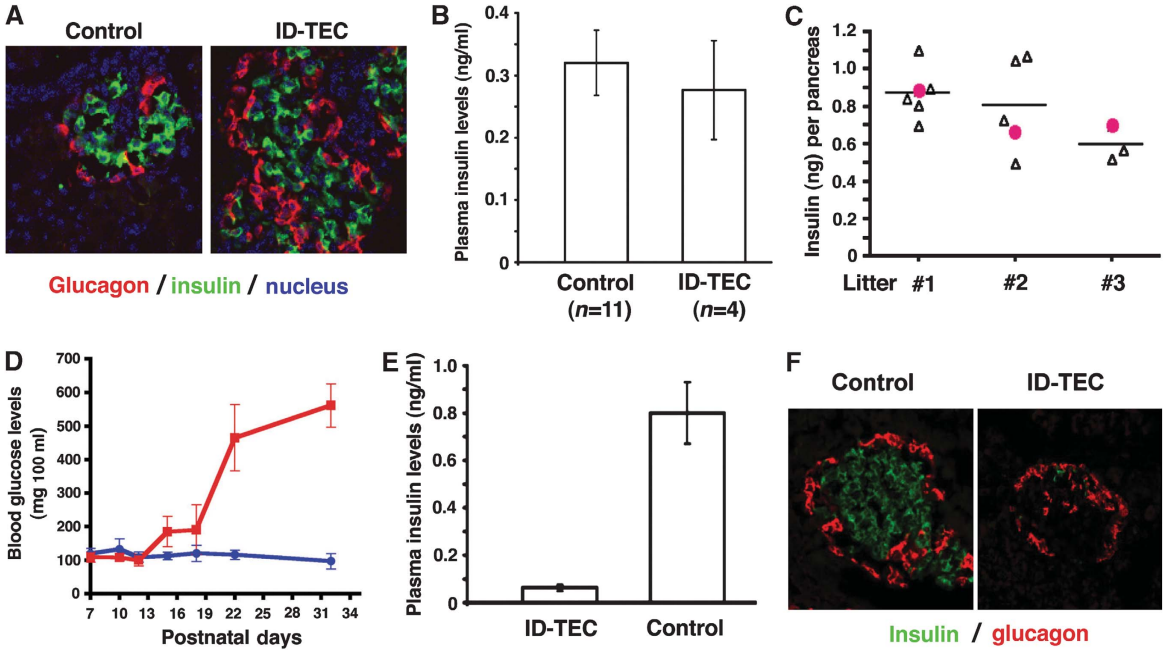

Figure 5 ID-TEC mice develop spontaneous diabetes within 3 weeks after birth. (**A**) Normal islet development of ID-TEC mice at birth. Pancreata from control and ID-TEC mice were collected at postnatal day 1, and stained using anti-insulin (green) and glucagon (red) antibodies. (**B**) Plasma insulin levels of 10-day-old ID-TEC pups. (**C**) Pancreatic insulin contents of 10-day-old ID-TEC pups. Pancreata were collected from three litters and insulin content in each pancreas was measured and plotted.—mean insulin levels; Δ, control littermates; filled red circle, ID-TEC pups. (**D**) Blood glucose levels of ID-TEC (red line, $n=7$) and control mice (blue line, $n=11$). (**E**) Plasma insulin levels of diabetic, 4-week-old ID-TEC mice. (**F**) Pancreatic sections stained with anti-insulin (green) and anti-glucagon (red) antibodies.

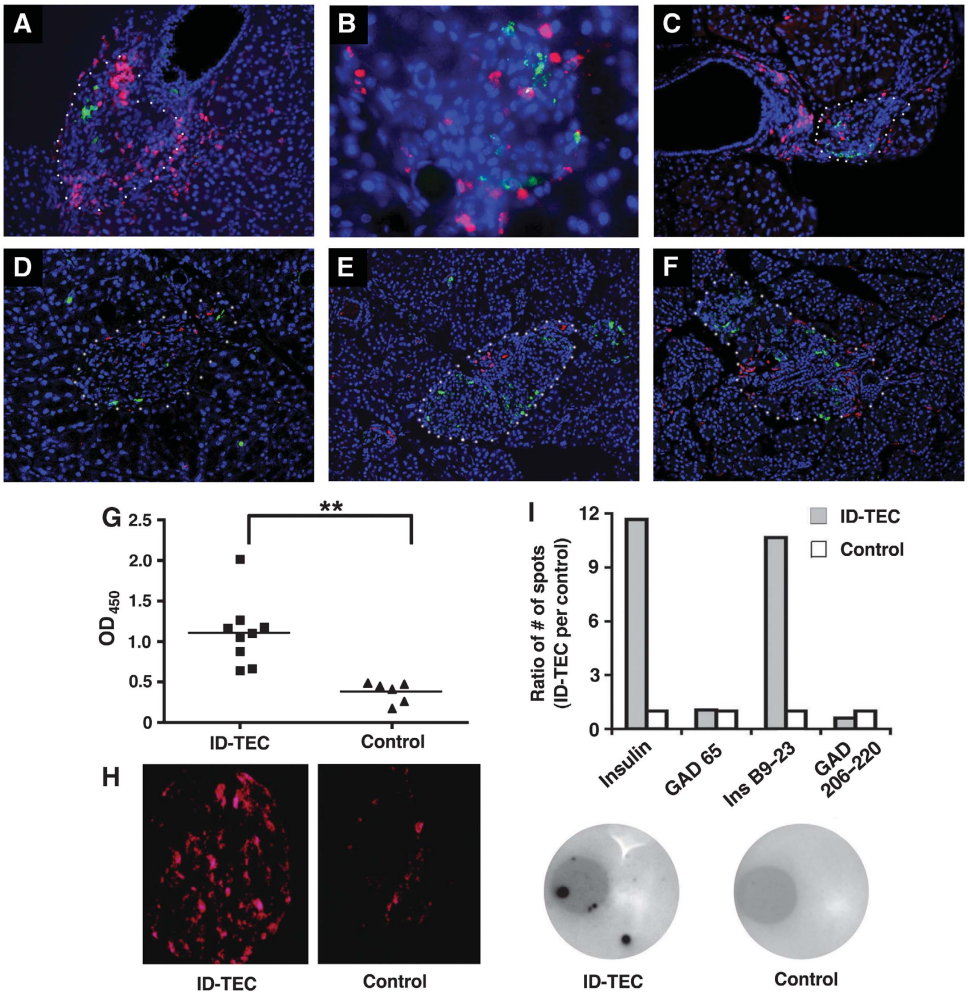

Figure 6 Diabetes developed in ID-TEC mice is autoimmune in nature. (**A**–**F**) Pancreata from ID-TEC mice were collected at postnatal day 21. Pancreatic sections were stained with anti-CD4 (red) (A–C), anti-CD8 (D), anti-B220 (E) and anti-F4/80 (F) antibodies (red). Higher magnification ($\times 400$) of a damaged islet with infiltrating T cells is shown in (B). Sections (A), (B), (D) and (F) were counter stained with anti-insulin antibody (green), whereas section (C) was counter stained with anti-glucagon antibody (green). Nuclei were stained with Hoechst 33342 in all the sections (blue). Damaged islets are outlined by dashes. (**G**) Sera collected from 3–8-week-old diabetic ID-TEC mice and age-matched controls (1:50 dilution) were subjected to insulin autoantibody (IAA) assay. Data are presented as absorbance at 450 nm (OD_{450}). —, mean value. $^{**}P<0.01$. (**H**) Sera collected from 6–8-week-old diabetic ID-TEC mice or control mice were used to stain islet sections from C57BL/6 *Rag1*$^{-/-}$ mice (1:5 dilution). Bound mouse antibody was revealed with goat anti-mouse IgG. (**I**) Presence of autoreactive T cells responding specifically to insulin in ID-TEC mice. Suspensions of splenocytes were pooled from three animals, and cultured overnight with whole insulin protein, whole GAD65 protein, insulin B-chain peptide 9–23 (Ins B9–23), or GAD65 peptide 206–220 (GAD 206–220). The frequency of IFN-γ-producing cells was measured by ELISPOT assays in triplicate. The upper panel shows one representative result from three independent assays. Filled bar, ID-TEC mice; open bar, controls. Representative microscopic pictures of ELISPOT assay using insulin as antigen are shown in the lower panel: ID-TEC splenocytes (left), controls (right).

in ID-TEC mice. Almost 50% of the spots were distinctly larger, indicating a high level of IFN-γ production by these T cells (Figure 6I). We also examined the insulin peptide B9–23, an immunodominant peptide found in both NOD mice and human T1D patients (Abiru *et al*, 2001; Alleva *et al*, 2001), and observed similar levels of response as those of insulin. In contrast, neither whole GAD65 protein nor its immunodominant peptide in NOD mice, peptide 206–220 (GAD 206–220), was able to stimulate a response at a level higher than littermate controls. The limited T cell response to the whole GAD protein and GAD 206–220 also suggests that epitope spreading had not yet occurred in ID-TEC mice. We were unable to detect any IL-4-secreting T cells using the same set of antigens, suggesting a primary TH1 response to insulin in ID-TEC mice (data not shown).

ID-TEC thymus is sufficient to induce spontaneous islet autoimmunity

To further show that the lack of insulin expression in thymic epithelial cells is sufficient to induce anti-insulin autoimmunity, we collected thymi from 2-week-old ID-TEC mice and transplanted them under the kidney capsules of athymic nude mice. At 16 weeks after thymus transplantation, the recipients were sacrificed and pancreata collected for histological evaluation. Flow cytometry analysis of lymph nodes and spleens obtained from thymus-transplanted recipients showed the success of T cell reconstitution (Figure 7A). Although no T cell infiltrates were found in pancreata from recipients of control thymi (Figure 7B), insulitis was readily observed in all pancreata collected from nude mice transplanted with the ID-TEC thymi (Figure 7C–E). These

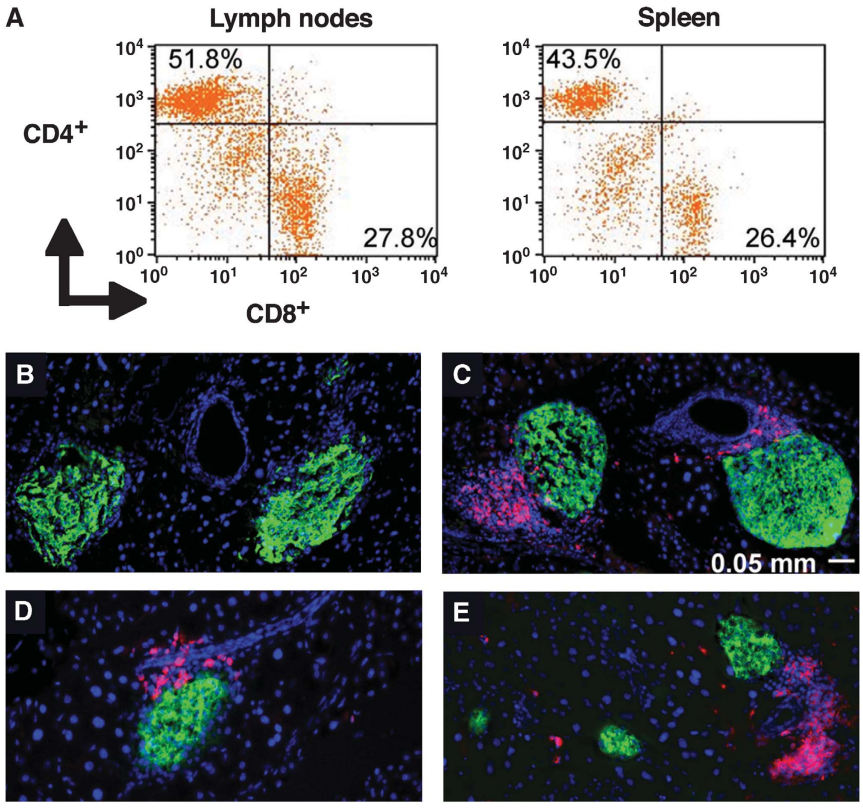

Figure 7 ID-TEC thymus is sufficient to transfer islet autoimmunity. (**A**) Representative FACS results showing the successful T cell reconstitution in nude mice with thymus transplantation underneath the kidney capsule. (**B–E**) At 16 weeks after thymus transplantation, pancreata were collected and stained with anti-CD4 (red) and anti-insulin (green) antibodies. (B) A representative pancreas section from nude mice transplanted with thymi from littermate controls, in which *Ins2* was intact, showing the absence of T cell infiltration. (C–E) Representative pancreata collected from nude mice transplanted with ID-TEC thymi.

experiments further showed the presence of thymus-derived, insulin-specific autoreactive T cell clones in the ID-TEC mice.

Islet autoimmunity can be transferred by both CD4$^+$ and CD8$^+$ T cells of ID-TEC mice

The autoreactivity of ID-TEC T cells towards islet β-cells was further shown by adoptive transfer experiments. Although limited by the number of cells that could be obtained from the extremely young mice, twenty million splenocytes pooled from a number of diabetic ID-TEC mice were infused into immune-deficient *Rag1* mice, which have the same histocompatibility background. At 1–2 weeks after the adoptive transfer, blood glucose levels of the recipients were elevated, compared with animals that received splenocytes from controls (205 ± 18 versus 127 ± 12 mg/100 ml, $n = 5$, $P < 0.01$, Student's *t*-test). Moreover, autoreactive T cells targeting insulin were found among the splenocytes collected from the adoptively transferred *Rag1* mice, as shown by ELISPOT assays for IFN-γ-producing cells (Figure 8A). The recipients remained modestly hyperglycemic for 2 months after the adoptive transfer and the presence of T cells in the proximity of the islets was readily detectable (Figure 8B). Taken together, these results showed that the T cell repertoire of diabetic ID-TEC mice includes cells that specifically recognized islet autoantigens, among them insulin or its peptides, although they were not able to develop a strong insulitis.

To further understand the functions that different groups of T cells had in mediating islet infiltration and destruction, T cells were isolated from the spleen and the lymph nodes of ID-TEC mice as effector CD4$^+$CD25$^-$ T cells and CD8$^+$ cytotoxic T cells, and transferred to *Rag1* mice either alone or together. At 4 weeks after the adoptive transfer, pancreata of *Rag1* mice were collected and analysed by immunohistochemistry. Insulitis and islet β-cell destruction were observed in *Rag1* mice adoptively transferred with either CD4$^+$CD25$^-$ or CD8$^+$ T cells, indicating that both cell types were capable of reacting to islet β-cells and eliciting an autoimmune response (Figure 8C and D). These results indicated that insulin-reactive T cells were present in both CD4$^+$ and CD8$^+$ T cell compartments in ID-TEC mice, and both types of T cells could home to the pancreas and cause damage to the islet β-cells.

ID-TEC mice displayed unimpaired immune tolerance towards autoantigens other than insulin

To exclude the possibility that our genetic manipulations cause generalized defects in central negative selection, we examined immune tolerance to male-specific HY autoantigens in ID-TEC mice. It is well established that thymocytes specific for HY minor antigens are negatively selected in the male thymus, whereas they can survive the selection process in the female thymus due to the absence of male antigen expression and presentation within the female thymic medulla (Simpson *et al*, 2002). Consequently, HY autoantigen-specific T cells were absent in the periphery of the male mouse, but can readily be detected in females on HY autoantigen challenge.

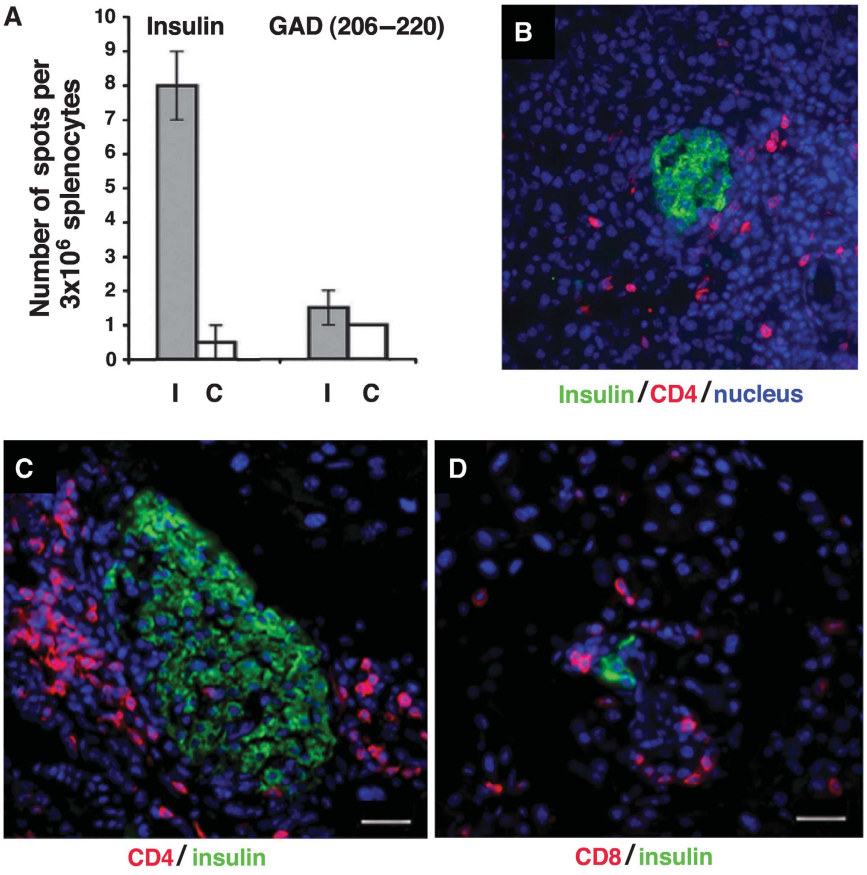

Figure 8 Both $CD4^+$ and $CD8^+$ T cells can transfer islet autoimmunity in immune-compromised *Rag1*$^{-/-}$ mice. (**A**) Anti-insulin autoimmunity can be transferred to C57BL/6 *Rag1*$^{-/-}$ hosts, which carry the same MHC haplotype ($H2^b$) as ID-TEC mice, by adoptive transfer of ID-TEC splenocytes. At 3 weeks after reconstitution, splenocytes were pooled from two animals, cultured overnight with either whole insulin or GAD 206–220 peptide as negative control. One representative result from three independent ELISPOT assays is shown. C, controls; I, ID-TEC (**B**) Detection of T cell infiltration in *Rag1*$^{-/-}$ pancreata after adoptive transfer of ID-TEC splenocytes. At 4 weeks after the transfer, pancreata were collected and stained with anti-CD4 (red) and anti-insulin antibody (green). (C, D) Pancreata were collected from *Rag1*$^{-/-}$ mice adoptively transferred with either $CD4^+CD25^-$ T cells (**C**) or $CD8^+$ T cells (**D**), and stained with anti-CD4 (C) or anti-CD8 (D) antibody (red). Insulin staining is shown in green.

Thus, examination of the presence of T cells specific to the HY autoantigens enabled us to evaluate the overall efficacy of thymic negative selection in ID-TEC mice.

We first examined the thymic expression of male-specific HY autoantigen expression in ID-TEC mice. As shown in Figure 9A, expression of genes encoding $H2^b$-restricted HY autoantigens (*Uty*, *Smcy* and *Dby*) was unaffected in the male ID-TEC thymus. Tetramer analysis of $CD8^+$ T cells specific to *Uty* (Millrain *et al*, 2001), one of the immunodominant HY autoantigens, showed that *Uty*-specific $CD8^+$ T cells were essentially absent in the spleens of male mice, but existed in large numbers in female spleens of both ID-TEC and control mice (Figure 9B). We also examined the presence of male autoantigen-specific $CD4^+$ cells in the spleens of both male and female ID-TEC mice through ELISPOT assays. The male-specific peptide, *Dby*, presented by MHC class II molecule ($I\text{-}A^b$), can stimulate IFN-γ secretion in numerous $CD4^+$ T cell clones within female spleens, whereas these clones were essentially absent in male spleens (Figure 9C). These data showed unimpaired negative selection of both $CD4^+$ and $CD8^+$ thymocytes specific to HY autoantigens in the thymus of male ID-TEC mouse, indicating that mTEC-specific insulin deletion does not generally compromise central selection, but is responsible exclusively for insulin-specific autoimmunity.

As impaired peripheral tolerance could also contribute to islet-specific autoimmunity, we also examined the presence of $CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{reg}) in ID-TEC mice. Pancreatic lymph nodes, mesenteric lymph nodes and spleens from ID-TEC mice were collected and analysed for the percentage of T_{reg} cells within the T cell population. Similar levels of T_{reg} cells were found in the $CD4^+$ T cell population in ID-TEC mice as in littermate controls, suggesting that the observed β-cell-specific immune destruction was not due to an overall quantitative deficiency in the T_{reg} compartment (Supplementary Figure S6).

To examine the functional capability of T_{reg} cells in suppressing the pathologic actions of autoreactive T cells in ID-TEC mice, we adoptively transferred $CD4^+CD25^-$ (T_{eff}) and $CD8^+$ (T_{cyto}) T cells into immune-deficient *Rag1* mice, with or without $CD4^+CD25^+$ (T_{reg}) cells. At 4 weeks after the transfer, pancreata of recipient *Rag1* mice were collected and analysed by immunohistochemistry. In striking contrast to the severe insulitis and islet β-cell destruction observed in *Rag1* mice adoptively transferred with T_{eff} and T_{cyto} cells

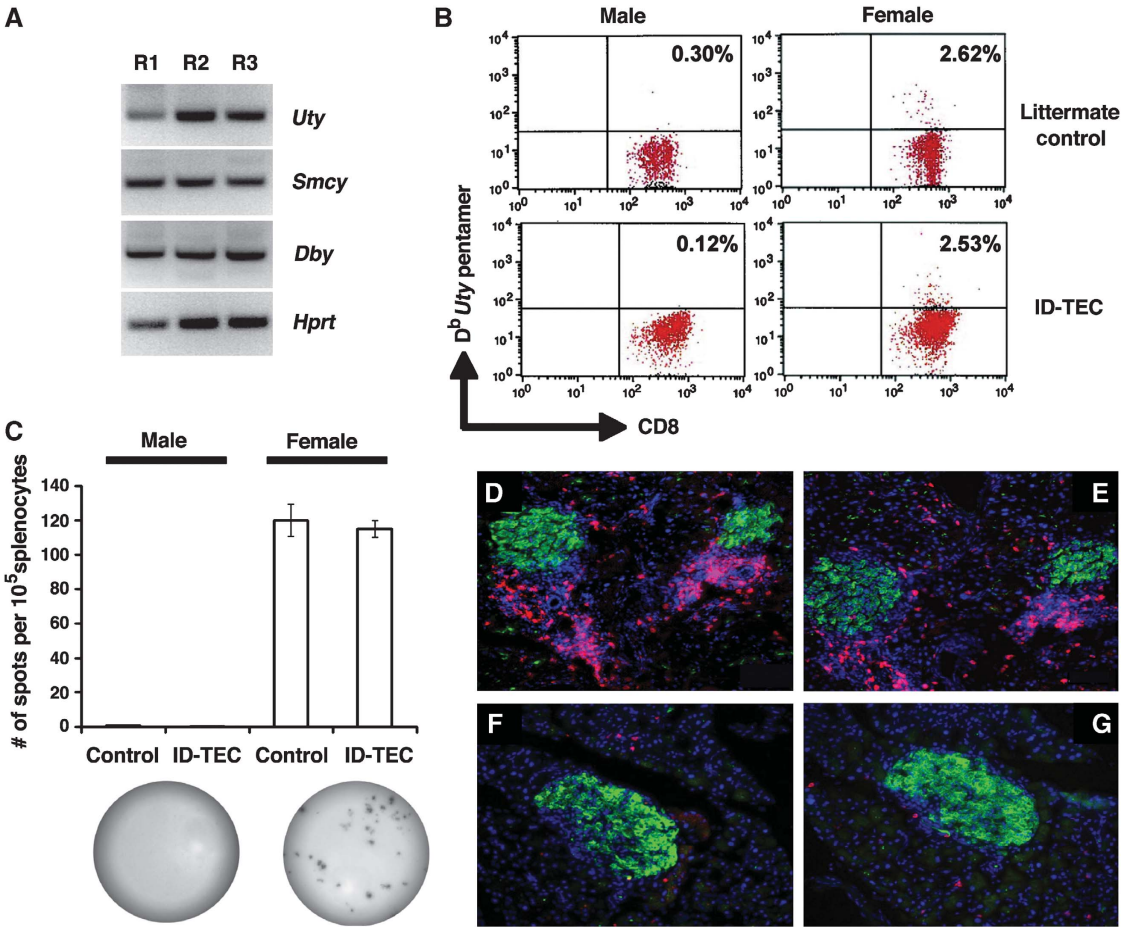

Figure 9 Unimpaired negative selection of HY autoantigens and normal T_{reg} cell development in male ID-TEC mice. (**A**) Thymi collected from male ID-TEC mice were separated as in Figure 1 (R1, thymic dendritic cells; R2, macrophage per B-cell; R3, mTEC). Isolated cells were subjected to RT–PCR analysis of expression of the genes encoding the immunodominant male-specific autoantigens (*Uty, Smcy and Dby*). (**B**) FACS analysis of *Uty*-specific $CD8^+$ T cells in the spleens of male and female ID-TEC mice. $CD8^+$ T cells were isolated from the spleens using magnetic beads and stained with anti-CD3 and anti-CD8 antibodies, together with D^b/Uty pentamers (ProImmune). Representative FACS data are shown, which were gated on the $CD3^+CD8^+$ population. (**C**) ELISPOT analysis of IFN-γ-secreting $CD4^+$ T cell clones stimulated with the immunodominant male-specific HY *Dby* peptide. Data are presented as mean ± s.e.m. Lower panel, representative microscopic pictures of the ELISPOT assay with male (left) or female (right) ID-TEC splenocytes are shown. (**D**, **E**). Pancreata collected from *Rag1*$^{-/-}$ mice adoptively transferred with $CD4^+CD25^-$ effector T cells and $CD8^+$ T cells, with (D, E) or without (**F**, **G**) an equal number of $CD4^+CD25^+$ T_{reg} cells, were stained with either anti-CD4 (D, F) or anti-CD8 (E, G) antibodies (red), counter-stained with anti-insulin antibody (green) and with Hoechst 33342 for nuclei (blue).

(Figure 9D and E), neither peri-insulitis nor insulitis was found when T_{reg} cells were co-transferred (Figure 9F and G), showing the suppressive capability of ID-TEC T_{reg} cells in inhibiting the autoimmune destruction of islet β-cells in adoptive transferred *Rag1* mice *in vivo*.

Discussion

Our results on ID-TEC mice established a direct link between thymic ectopic expression of a single tissue-specific gene (i.e., insulin) and the development of a self-tolerant T cell repertoire towards specific peripheral tissues. Such a correlation was shown in immunologically naïve animals, without any manipulation of the natural developmental process of the immune system, or disturbance to the overall thymic negative selection of autoreactive T cells specific to other autoantigens. Moreover, full-blown, spontaneous, autoimmune diabetes develops in ID-TEC mice around 3 weeks after birth. This is in striking contrast to the previously described *Ins2* total knockout mice, in which mild anti-insulin autoimmunity occurred only after immunization with the (pro)insulin-2 molecule, with neither pathological destruction of β-cells nor T cell infiltration into the pancreas (Faideau *et al*, 2006). Although thymic expression of (pro)insulin-2 molecules was reduced in both mouse models, the presence of functional alleles of the *Ins1* gene, even with its limited expression in the thymus, might explain the only partial breakdown of insulin tolerance observed in the *Ins2* total knockouts. In agreement with Faideau *et al* (2006), ID-TEC control littermates with even one functional *Ins1* allele (genotype Aire–Cre:*Ins1*$^{+/-}$:*Ins2*$^{l/l}$) remained euglycemic and showed no sign of insulin autoimmunity through life (our unpublished observation). The *Ins1* knockout in the genetic background of our ID-TEC mice avoids these pitfalls. Our data clearly show that in the absence of *Ins1*, depletion of *Ins2* expression in the thymus is sufficient to impair negative selection in ID-TEC mice, resulting in the migration of Ins2-specific, autoreactive T cells out of the thymus. Under these circumstances, autoantigen(s) derived from (pro)insulin-2 molecules was sufficient to drive the autoimmne response

of Ins2-specific T cells in the periphery, causing the progressive destruction of pancreatic β-cells, even in the absence of any evident environmental trigger or manipulation.

In theory, there are a number of ways that will allow insulin epitopes to be presented to T cells in the thymus to mediate central tolerance: bone-marrow-derived thymic APCs and macrophages can take up and transport circulating insulin molecules to the thymus (Li *et al*, 2009); APCs and macrophages could express (pro)insulin ectopically in the thymus (Pugliese *et al*, 2001); endoderm-derived TECs, especially those within the medullary region, express and present to the T cell directly (Faideau *et al*, 2006); or peptides derived from the insulin molecules, produced from mTEC, could be cross-presented by bone marrow-derived APCs to mediate the deletion of the autoreactive T cells within the thymus (Surh and Sprent, 1994). Our data clearly showed that circulating insulin was not effective in mediating negative selection of Ins2-specific T cells in our young animals, as no statistical difference of either pancreatic insulin contents or circulating insulin levels was detected between ID-TEC pups and control littermates at birth and by postnatal day 10. Furthermore, it was previously shown that the efficacy of antigen presentation by dendritic cells increased, at least, 100–1000-fold when an anti-DEC-205 antibody-based strategy was used to facilitate the antigen internalization into the target cell as compared with natural uptake from the circulation (Hawiger *et al*, 2001; Dudziak *et al*, 2007; Mukhopadhaya *et al*, 2008). Thus, it is conceivable that expression in mTECs could be more efficient, or even indispensable, for effective autoantigen presentation to generate a self-tolerant T cell repertoire. Although we did not observe the presence of *Ins2* transcripts in bone marrow-derived thymic APCs under our experimental conditions (Figure 1B), the residual ($<10\%$) insulin transcripts observed in the ID-TEC thymus might come from these bone marrow-derived cells under the hyperglycemic conditions of the diabetic ID-TEC mice. Indeed, there are a number of reports showing elevation of ectopic insulin expression in bone marrow-derived cells under these abnormal conditions (Kojima *et al*, 2004; Carlsen and Cilio, 2008). Furthermore, overexpression of autoantigens, including insulin, in bone marrow-derived APCs has been shown to attenuate or prevent autoimmune diabetes progression in NOD mice. Whether this regained insulin ectopic production in the immune cells could re-establish central and/or peripheral tolerance remains to be investigated, and the ID-TEC mice would serve as an ideal experimental model.

Ins2 transcripts were also drastically downregulated in the thymus of *Aire* total knockout mice, but only a portion of animals showed anti-islet autoimmunity, with no report of pathologic destruction of pancreatic β-cells (Anderson *et al*, 2002). Our Cre–Lox approach is likely to be more effective at eliminating *Ins2* expression in specific *Aire*-expressing thymic cells. Furthermore, thymic *Ins1* expression in the *Aire* knockout mice might help to eliminate insulin-specific autoreactive T cells, as it is not affected by Aire expression. In addition, differences between *Aire* total knockouts and our ID-TEC mice could also result from other important aspects of *Aire* function, such as control of the interactions between T lymphocytes and TECs, or the regulation of TEC differentiation (Cheng *et al*, 2007; Gillard *et al*, 2007). Nevertheless, our data show that the negative selection checkpoint is facilitated by insulin produced by *Aire*-expressing, endoderm-derived mTECs, although bone marrow-derived cells in the ID-TEC mice may also have a function by cross-presenting insulin peptides expressed in thymic epithelium (Surh and Sprent, 1994; Gray *et al*, 2007), or picked up from circulation (Li *et al*, 2009).

Recently, it was found that Aire is also expressed in a subset of $CD45^-$, MHC II^+, $EpCAM^+$ stromal cells (defined as extrathymic Aire-expressing cells, eTACs) in the peripheral lymphoid organs (Gardner *et al*, 2008). Autoantigen expression in eTACs could enable the peripheral deletion of autoreactive T cells. This interesting new aspect of the *Aire* gene places it in a pivotal position in regulating the gene expression network essential for both central and peripheral tolerance. However, there was little overlap observed between the set of Aire-regulated genes in medullary TECs and eTACs, and insulin was not among the genes found to be influenced by Aire in eTACs in the periphery. Similar to their Aire-driven *Adig* transgene, our *Aire–Cre* transgene is also expressed at very low levels in spleen. It remains to be determined whether *Aire–Cre* is also expressed in eTACs to mediate *Ins2* deletion in peripheral tissues and its potential immunologic significance. Regardless, our thymus transplantation data clearly demonstrated the dominant role of central insulin expression in regulating islet autoimmunity, as ID-TEC thymus alone is sufficient to generate insulin-reactive, islet-infiltrating T cells, even though neither *Ins1* nor *Ins2* gene was affected in peripheral lymphoid tissues.

Similar to NOD mice, immune cells of both adoptive and innate immunity were found infiltrating the pancreatic islets. However, we did not observe the signature massive T lymphocyte infiltration (i.e. peri-insulitis and insulitis) in our adoptive transfer experiments, like that classically seen in pre-diabetic NOD mice and in humans. One distinct difference is the early, rapid onset of the islet destruction in our ID-TEC animals. The chronic disease progression in NOD mice provides an opportunity for the amplification of the inflammatory process by epitope spreading and bystander effects. However, it has been suggested that a small number of active effector cells may be sufficient to destroy the β-cells of an islet (von Herrath and Homann, 2004). Thus, the classic image of insulitis may reflect an expanded, less specific process occurring after the initial islet β-cell damage; a later phase that we simply did not observe, perhaps also due to the autoimmune resistant $H\text{-}2^b$ MHC of the ID-TEC animals in comparison with the autoimmune prone $H\text{-}2^{g7}$ allele of the NOD mice (von Herrath and Nepom, 2009). The large spot sizes observed in ELISPOT assays of IFN-γ production indicate the strength of the TH1-type response developed once the insulin-reactive T cells are stimulated (Hesse *et al*, 2001). In the ID-TEC mice, this strong response mediated by a small number of cells may cause pathological damage to islet β-cells at an earlier age. Consistently, islets with severe damage could be found in *Rag1* mice transplanted with, as little as, 5×10^5 $CD8^+$ T cells from ID-TEC mice. Islet destruction accompanied with massive T cell infiltration was also detected in $CD4^+CD25^-$ effector T cell-transplanted *Rag1* mice. Thus, as the consequence of abolished thymic insulin expression, insulin-reactive T cells are present in both $CD4^+$ and $CD8^+$ compartments in ID-TEC mice.

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 T_{reg} cells (Monteiro *et al*, 2008). Within the neonatal environment, establishment of proper T_{eff}:T_{reg} ratio is crucial to prevent autoimmune disease later in adult life. Indeed, delay of T_{reg} migration from the thymus through 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 substantially higher number of insulin-specific T cells, which could disturb the proper ratio of T_{eff}:T_{reg} cells. This unchecked expansion of insulin-reactive effector T cells could inflict irreversible damage to the β-cells before the expansion of T_{reg} 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 β-cell apoptosis during the 2nd and the 3rd 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 APCs and initiated insulitis in pre-diabetic NOD mice. As a high-level expression of the *Aire–Cre* transgene was observed in the newborn ID-TEC thymus (Y Fan *et al*., 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 3rd postnatal week, when signs of islet inflammation are present. Thus, β-cell apoptosis might have an indispensable function 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 mutual exclusively of the above mechanisms, other peripheral self-antigen tolerance mechanisms could limit the progression of islet autoimmune destruction.

In the ID-TEC mouse model, the ability to negatively select insulin-specific autoreactive T cells within the thymus is undermined by the total absence of the self-antigen expression, even in the presence of disease-resistant H-2^b MHC molecules. Our data support the conclusion that (pro)insulin is a major autoantigen for the initiation and/or progression of T1D, as previously hypothesized from studies of clinical cases, suggesting the association of diabetes susceptibility with genetic differences that affect insulin expression (Walter *et al*, 2003). Our data can also explain the rare cases of T1D with rather early onset in children expressing protective HLA alleles. The ID-TEC mice could serve as a new model to study this particular type of T1D, as both male and female ID-TEC mice develop spontaneous diabetes around 3 weeks after birth with 100% penetration, in striking contrast to the NOD animal model in which 80–90% females develop the disease over a period of 20 weeks (from 12 weeks to 40 weeks), a gender preference not observed in human T1D cases. Our study also suggests that the functionality of transcription factors able to regulate thymic self-antigen expression should be examined in young patients to help diagnosis. Disease prevention strategies may need to address the antigenic role of insulin in the development of T1D.

Materials and methods

Mice

Loxp-tagged *Ins2* mice were generated through standard gene targeting methods as described by Fan *et al* (1999). *Aire–Cre* transgenic mice were obtained through pronuclear injection of the linearized Cre construct into fertilized eggs collected from C57BL/6 mice. Transgenic lines were subjected to multiple rounds of breeding with $Ins1^{-/-}$:$Ins2^{l/l}$ mice to obtain ID-TEC mice. Thus, all the animals in the study are on the 129:B6 mixed genetic background, with the MHC H-2^b haplotype shared by both the 129 and C57BL/6 strains.

Heterozygous *loxp*-tagged Ins2 mice were first crossed to Flp recombinase-expressing mice (129S4/SvJaeSor-$Gt(ROSA)26Sor^{tm1(FLP1)Dym}$/J, Jackson Laboratory, Bar Harbor, ME) to delete the *Neo* cassette, and then interbred to obtain homozygous $Ins2^{l/l}$ mice. Subsequently, $Ins2^{l/l}$ mice were crossed to *Ins1* knockout mice (a gift from Dr Jacques Jami) to obtain $Ins2^{l/l}$:$Ins1^{-/-}$ mice.

Immune deficient C657BL/6 $Rag1^{-/-}$ mice (B6.129S7-$Rag1^{tm1Mom}$/J), Rip–Cre mice (B6.Cg-Tg(Ins2-Cre)25Mgn/J) and Rosa26R-lacZ (B6.129S4-$Gt(ROSA)26Sor^{tm1Sor}$/J) mice were all purchased from the Jackson Laboratory, Bar Harbor, ME. All mice were housed in a specific pathogen-free animal facility at Rangos Research Center, Pittsburgh. All experiments were carried out in accordance with institutional guidelines.

Constructs

Detailed procedures to obtain the constructs used in the study (the *Ins2* targeting construct and the *Aire–Cre* construct) are available on request. Briefly, to constitute the *Aire–Cre* plasmid, DNA segments flanking the Aire-coding region were subcloned from a BAC plasmid containing the mouse Aire gene. The *Aire–Cre* construct was assembled from a 23-kb DNA fragment containing the sequence 5′ of the mouse *Aire* gene and including its first exon, a PCR product of the Cre recombinase cDNA, and an 11-kb fragment 3′ of the last exon of the *Aire* gene.

Blood glucose and insulin levels

Blood glucose levels were monitored using the Ascensia Contour blood glucose monitoring system (Bayer HealthCare LLC, Mishawaka, IN). Blood insulin levels were measured using the Mercodia Ultrasensitive Mouse Insulin Elisa Kit (Mercodia AB, Sweden), following the manufacturer's protocol.

RNA analysis

The total RNA of individual thymi or pooled thymi was isolated using an RNA minikit, according to the manufacturer's protocol (Qiagen). Real-time PCR was carried out on cDNA prepared from DNase I-treated RNA (Superscript III cDNA kit, Invitrogen) using the LightCycler FastStart DNA Master SYBR Green I kit, and analysed using the LightCycler 2 system (Roche Applied Science).

Primer pairs used in the study

Hprt:	F 5′-GGATACAGGCCAGACTTTGTTGGA-3′
	R 5′-CAACAGGACTCCTCGTATTTGCAG-3′
Aire:	F 5′-AATCTCCGCTGCAAATCCTGCTCT-3′
	R 5′-ACTGCAGGATGCCGTCAAATGAGT-3′
CD45:	F 5′-AGGACCTCAAACAGAAGCTTCCCA-3′
	R 5′-TTGCAGAGAGGCCTGGAGATCTTT-3′
Cytokerain 2/8:	F 5′-AGGAGCTCATTCCGTAGCTG-3′
	R 5′-TCTGGGATGCAGAACATGAG-3′
Cre:	F 5′-TCGCTGGCGTTTCTATGACGAGAA-3′
	R 5′-ATCACTCGTTGCATCGACCGGTAA-3′
Insulin:	F 5′-CACCCAGGCTTTTGTCAA-3′
	R 5′-AGATGCTGGTGCAGCACT-3′
β-actin:	F 5′-TGGAATCCTGTGGCATCCATGAAA-3′
	R 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

Histology

Pancreata and thymi were collected, fixed in 4% paraformaldehyde for 3 h at 4°C, and placed in 30% sucrose overnight. Cryosections,

5 μm thick, were cut and stained using primary antibodies. Antibodies used in the study: CD3, CD45, CD4 and CD8 (BD Biosciences, Franklin Lakes, NJ); insulin (Santa Cruz Biotechnology, Santa Cruz, CA); glucagon (Zymed, San Francisco, CA); β-galactosidase (Chemicon, Temecula, CA).

Adoptive transfer experiments

Spleen, mesenteric and pancreatic lymph nodes were collected from ID-TEC mice. Single cells were prepared and subjected to magnetic bead isolation first for $CD4^+$ T lymphocytes following manufacturer's protocols (Miltenyi Biotec, $CD4^+$ T cell isolation kit), followed by FACS sorting of $CD25^-$ and $CD25^+$ cells. Purities above 95% were achieved routinely. The $CD8^+$ T cell isolation kit was used for $CD8^+$ T cell isolation (Miltenyi Biotec). Equal numbers (5×10^5) of T cells of the different subsets were mixed and injected into *Rag1*$^{-/-}$ mice through the tail vein (i.v.).

ELISPOT assay

ELISPOT assays were carried out using the BD mouse IFN-γ ELIPOT set, according to the manufacturer's specifications (BD Biosciences). Splenocytes (3×10^6) from 2–3 mice were pooled and cultured overnight with 0.1 mg/ml insulin (Sigma-Aldrich), whole GAD65 protein (Abnova), insulin B chain peptide 9–23 (SHLVEALYLVCGERG, AnaSpec), GAD65 peptide 206–220 (TYEIAPVFVLLEYVT, Sigma-Aldrich) or medium alone. Assays were repeated and carried out in triplicate and averaged. To evaluate immune response to male-specific HY autoantigen, 2×10^5 splenocytes were incubated with 10 ug/ml *Dby* peptide (NAGFNSNRANSSRSS, AnaSpec) overnight.

Thymus transplantation

Thymic lobes were isolated from 2-week-old ID-TEC pups or littermate controls, and cultured in transwells at 37°C in the presence of 1.35 mM deoxyguanosine (Sigma-Aldrich) for 10 days to eliminate hematopoietic cells. Two lobes were grafted beneath the kidney capsule of each 6–8-week-old nude mouse. At the end of the study (16 weeks post thymus transplantation), pancreata were collected for histological analysis.

Tetramer analysis of $CD8^+$ T cells specific to HY autoantigen Uty

Animals were injected with 5×10^5 male splenocytes collected from littermate controls of ID-TEC mice intraperitoneally (IP). At 2 weeks after the initial priming, splenocytes were separated using $CD8^+$ T cell isolation kit (Miltenyi Biotech) and stained with CD3, CD8 and Pro5 MHC class I D^b/*Uty* pentamer, following manufacturer's protocols (ProImmune).

Insulin autoantibody (IAA) assay

A 96-well EIA plate was coated with 2 μg of heat-inactivated (95°C, 30 min) human insulin in 100 μl of PBS overnight. Wells were blocked with PBS with 10% BSA for 2 h at room temperature, and probed with mouse sera collected from 3–8-week-old ID-TEC mice and age-matched controls (1:50 dilution, 100 μl in each well) for another 2 h. Next, biotin-conjugated rat anti-mouse IgM (BD Biosciencs) was added to the well (1:5000 dilution) and incubated for 30 min, followed by incubation with horseradish peroxidase-conjugated streptavidin (1:4000) for 15 min. Lastly, ACE substrate (BD Biosciences) was added to each well and the absorbance was measured at 450 nm using Bio-Rad Microplate Reader.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank R Lakomy and A Stychie for help with FACS sorting and J Flounlacker for assistance with the mouse colony maintenance. We also thank J Jami and C Polychronakos for the gift of the *Ins1*$^{-/-}$ mice. This work was supported, in part, by the Henry Hillman Endowment Chair in Pediatric Immunology and by a Department of Defense grant W81XWH-06-1-0317 (MT), and by a Cochrane Webber Endowment (YF).

Conflict of interest

The authors declare that they have no conflict of interest.

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Pediatric Diabetes

Pediatric Diabetes 2010: 11: 292–295
doi: 10.1111/j.1399-5448.2010.00704.x

Pediatric Diabetes

Perspective

Beta-cell regeneration: from *science fiction* to challenging reality

Type 1 diabetes is due to an autoimmune reaction directed against the pancreatic beta cells. Since some regenerative capabilities of the endocrine pancreas have now been quite well documented, and recent research has shown that human stem cells can be derived from embryos or from genetically engineered somatic cells, is it practical or even possible to combine these lines of research to more effectively treat young diabetic patients? The recently published paper of Pedro Herrera and his group (1) inspires a number of considerations that might help to properly answer this question, while bringing to mind unforgettable scenes from a popular 90's film. Herrera's results show that after near-total ablation of beta cells in the endocrine pancreas, some new beta cells are actually generated. However, these new beta cells originate from already existing alpha cells, rather than from preexisting beta cells or from other precursor cells.

> *Metallic drops, warmed by the sparks of melting iron, quiver and flow on the ground like quicksilver. They coalesce, and together they expand, change conformation, and eventually assume their final shape: it's T-1000, the liquid metal-based robot in the famous movie "Terminator 2" directed by James Cameron. The T-1000 is the nemesis of the T-800, the other, more conventional robot, sent back in time as mankind's savior. The task for the T-800 is quite difficult, since the T-1000 seems to be indestructible: Even after enduring tremendous damage, it can easily reassemble, repair, and finally regenerate itself, quickly resuming its original form.*

Fortunately for us, we do not have to be overly envious of the T-1000. Once damaged, parts of the human body also have the ability to repair, reassemble, and regenerate themselves, ultimately resuming their original conformations. With the exception of only a few tissues, our cells can be replaced fairly quickly and regenerate into functional tissues. Perhaps we should not be surprised, then, that the endocrine pancreas is capable of functional regeneration, given adequate time and the appropriate physiological conditions. However, the nature of the requisite beta cell precursors, how they function, where they are physically located, and what influences them to regenerate the correct missing tissue, are all questions still awaiting definitive answers.

The 'stem cell' is, by definition, the one cell capable of duplicating itself and while maintaining its undifferentiated status, could also originate a progeny that differentiate into one or more different final products that are physiologically defined by their specific functions. Proceeding through the differentiation pathway, stem cells can be categorized as totipotent, pluripotent, multipotent, oligopotent, and unipotent, depending upon all their possibly irreversible, progressively acquired characteristics. Twins can actually develop from the same zygote, if its derived cells are properly separated very early in the embryological process. Although then, the vertebrate zygote might be considered the preeminent totipotent stem cell, it continues to divide to form the final individual: a mixture of similar, but not identical, daughter cells. Since we do not know yet which specific markers characterize the totipotent cell, we also do not know whether totipotent cells are still preserved, once the various tissues begin to differentiate into organs. If they are, how long could they continue to be functional? Intuitively, we can argue that precursors of some kind should still be present and active within the body indefinitely, because even elderly people are able to repair their damaged tissues. However, we still do not know the number of these regenerative precursors, where they may be hiding, and which level of differentiation they have already achieved.

Paraphrasing what I wrote some years ago (2), I would argue that a system based on a single regenerative center, serving all the peripheral needs, would be quite inefficient. If this were the case, even if an 'S.O.S.' transmitted from the periphery traveled quickly, the center, once alerted, would need too much information and too much time to generate

the specific precursors. In other words, the center would not be able to deliver an adequate number of the appropriate cells to the 'scene of the crime' quickly enough to avoid disaster. Therefore, to be most effective, these hypothetical regenerative centers would have to be scattered throughout the body. This is the same rationale for stationing firehouses throughout an entire city, to allow each unit to be able to more rapidly reach any fire location and efficiently intervene.

Our sophisticated regenerative system does not need to deploy totipotent cells into each organ. It would be enough to maintain in each organ sufficiently undifferentiated precursors with self-maintenance capabilities, as well as those necessary to replace the worn-out cells of the organ. Also desirable would be a physiological process that progresses relatively slowly to maintain tissue homeostasis but which could be converted, in the case of crisis, into a rapidly operating system. Consequently, this system would be highly effective at responding promptly to abruptly received, alarming feedback signals.

In the endocrine pancreas as well, long-lasting insulin-producing beta cells should be continuously, albeit quite slowly, replaced by newly generated cells. At the time of need, besides the possible replicative ability of the beta cells themselves (3), other regenerated cells should come from precursors, possibly located among pancreatic ductal cells (4). Thus, these regenerative units would be in close physical proximity to the endocrine tissue, the islets of Langerhans. When the pancreas is physically damaged, the pace of the physiologic reparative process accelerates and a more evident regeneration product can be observed.

This actually seems to be the message we receive from the recently published paper of Herrera's group (1) in which the inherent regenerative capacity of the adult pancreas to produce new beta cells was systematically studied. To generate a strong enough 'danger signal', an extreme situation was created in which near-total beta cell loss can be obtained at will to mimic type 1 diabetes pathology, but in the absence of autoimmunity. For this purpose, the authors used two *in vivo* genetic approaches: cell ablation combined with cell lineage tracing. Inducible, rapid cell removal (>99%) was obtained by administration of diphtheria toxin (DT) in transgenic mice in which the diphtheria toxin receptor (DTR) was expressed by the beta cells only. The systemic administration of DT permits an exquisite, specific ablation of almost all existing beta cells by apoptosis. Newly formed beta cells were easily monitored using a reliable cell lineage tracing. The results obtained surprisingly showed that the adult pancreas can actually generate new beta cells after their near-total loss, but mainly by reprogramming its glucagon-secreting alpha cells.

Taking all this information together, a physiologic scenario can be then envisaged in which tissue-specific precursors, present among pancreatic ductal cells (4), are generating alpha cells and beta cells (2). Beta cells, in turn, even if extremely slowly, replicate themselves to maintain homeostasis (3). However, once stimulated by a powerful danger signal, glucagon-positive alpha cells can then transdifferentiate into insulin-secreting beta cells to repair the damage (1).

In humans, without the help of lineage tracing, specific markers become necessary to recognize and eventually physically isolate tissue-specific precursors. However, even if these markers were already utilizable, intuitively one sees that isolating precursor cells from a patient's own pancreas would not be an easy task. Increasing their numbers *ex vivo* while avoiding the activation of differentiation pathways would also be problematic, as would facilitating their differentiation toward the wanted final product. Here again James Cameron anticipated a possible solution of the problem.

> *The T-1000, like an exceptional chameleon, blends into any scene, assuming the most appropriate appearance suggested by each environment it encounters. In the film, the T-1000 completely hides itself, becoming part of the black and white tiles of the floor of a hospital ward. It then regenerates from the linoleum into a human form. However, it does not resume its original human appearance; instead, it takes on the appearance of the security guard on duty where the patient, for whom it is looking, is unwillingly detained.*

Faithfully recreating the environment necessary to guide and facilitate a desired type of differentiation *in vitro* appears to be quite difficult. An easier solution might be to somehow isolate and then physically introduce a precursor into the already existing, appropriate environment like, e.g., the embryonic pancreas (5). The precursor would then be allowed to get 'acquainted' with its new surroundings by assuming the most appropriate appearance (i.e., phenotype) to better fit the new context. By becoming one with the new environment, the precursor may quickly convert into the product best equipped to repair the damage. The signals sent through host-secreted factors or by cell-to-cell contacts seem to be powerful enough to guide the differentiation process toward the most needed product, even across different lineage barriers. This ability to 'transdifferentiate' (i.e., generate a progeny belonging to a tissue lineage different from the one of origin) certainly is an astonishing discovery. A few years ago, no one would even speculate that a mammalian stem cell,

present in an adult individual, could still possess such an impressive plasticity. The possibility that new beta cells could be generated from 'adult' stem cells even passing through an alpha cell phenotype (1) offers a particularly appealing alternative, because it avoids the potential ethical problems associated with the use of embryonic stem cells.

However, even assuming the existence of these beta cell-specific precursors, we still do not know whether they are immortal or are actually subject to senescence, leaving us perhaps with a narrow window for intervention. This aspect may be especially relevant in diabetic individuals in whom the reparative process has been kept under check by autoimmune patrolling for a long period of time. In a case in which prompt intervention (e.g., immediately after the clinical onset of the disease) is not possible, would we still be able to repair the already 'seasoned', deleterious damage of the endocrine pancreas? In the absence of precursors, once they all might be already dead, which resources can still be used?

> *The T-800 is abruptly catapulted into a foreign environment and another time period. Under the friendly guidance of a young boy, who patiently introduces human feelings, behaviors, and vernacular expressions into its newly accessible electronic memory, the T-800 assimilates this passively received and progressively accumulated information. Eventually it becomes able to interact in a more meaningful way with its surroundings. As a direct consequence, the T-800 starts experiencing human-like feelings and becomes so sensitive to human concerns that it decides on self-immolation as the only path that will halt or delay the destruction of the world it has begun to comprehend.*

Many remarkable results have been obtained in the research laboratory by transfecting cells of a certain lineage (e.g., fibroblasts) with genes (i.e., *Oct4*, *Sox2*, *Klf4*) encoding different transcription factors able to convert these somatic cells into ones carrying the characteristics of pluripotent precursors. The human induced pluripotent stem (iPS) cells, in turn, could differentiate into cells of a different lineage, like insulin-producing cells, even if the quantity in which these precursors can be reliably generated seems to be quite limited (6). Furthermore, assuming that we could overcome these limiting aspects, the possibility of using human stem cell lines tailored *ad personam* is certainly revolutionary, even if it were quite inefficient, frequently unsuccessful, and consequently extremely expensive. This approach, while allowing us to bypass the big problem of allorejection, simultaneously opens the door to the possibility that these dividing cells will not stop growing once a specific, predetermined mass has been reached. In the situation in which these cells do not spontaneously stop proliferating, we could have, unknowingly and tragically, transplanted cancer precursors into our patients.

Finally, assuming that we would be able to establish pluripotent stem cell lines for each patient, and eventually derive from them a specific progeny with the correct phenotype, and that we could generate a sufficient number of the missing beta cells to satisfy the needs of the diabetic recipient, we would still have to solve the problem of recurrent autoimmunity. In patients with type 1 diabetes, autoimmunity not only damages the original endocrine tissue, bringing them to the clinical onset of the disease, but also efficiently limits its reparative process. In fact, autoreactive, diabetogenic T-cell clones, escaped from thymic control (7), seem to be able to systematically kill newly generated beta cells with which the precursors try to replace the ones that are lost. Once transplanted into diabetic mice or humans, syngeneic, healthy beta cells are quickly killed by these same perpetrators, namely $CD8^+$ effector T-cells (8).

This autoimmune process is successfully annihilated in the diabetes-prone [e.g., the non-obese diabetic (NOD)] mouse either by substituting all or a part of the immunocompetent cell pool of the recipient with bone marrow cells from a diabetic-resistant donor. Complete substitution is accomplished with conventional bone marrow transplant techniques, and partial substitution by establishing hematopoietic chimerism (9, 10). If these processes were to proceed in humans, they should be sufficient to ensure the recipient's well-being long enough to let him or her benefit from the positive consequences of the laborious, regenerative process. However, more important is the consideration that the successful engraftment of the transplanted bone marrow, or the establishment of a steady hematopoietic chimerism, would have to be obtained and eventually maintained without the use of immunosuppressive agents. This is because these potent drugs, by definition, would kill not only the immunocompetent cells of the recipient but also the beta cells themselves, which are particularly sensitive to the toxicity of the immunosuppressive agents (11). This would, of course, completely defeat the purpose of the transplant.

Perhaps more easy to implement is another approach successfully used in the mouse to stop autoimmunity. Dendritic cells (DCs) are the body's sentinels largely responsible for host surveillance against microenvironmental anomalies including pathogen invasion, infection, and damaged tissue architecture. In a functionally immature state (characterized by low to absent expression of costimulatory molecules such as

CD40, CD80, and CD86), DCs are powerful agents of immune hyporesponsiveness. Exogenous administration of functionally immature DCs achieves long-term and stable allograft survival in a variety of mouse and rat models and prevents a number of autoimmune diseases. Mechanistically, functionally immature DCs act by inducing anergy of the dangerous effector T-cells, either via direct cell contact and/or cytokines and by upregulating the number and function of regulatory T-cell subsets. It has also been shown that *in vitro* administration of nuclear factor-kappa B (NF-kappa B) decoys to DC as well as direct targeting of CD40, CD80, and CD86 with antisense oligodeoxyribonucleotides (AS-ODNs), reduce costimulatory molecule expression levels, producing functionally immature DCs capable of preventing or even reversing new-onset diabetes, once reintroduced into the diabetic recipient, the same NOD mouse from which they were originally collected (12). If we would be able to somehow stop autoimmunity, then it would be sufficient to characterize the factors able to trigger the regenerative process of the beta cell, allowing us to bypass quantitative and chronological limits of resident precursor cells and to reconstitute in this way an efficient glycemic control in our young patients (13).

Diabetic patients must check their blood glucose levels and be injected with insulin at least four times a day. Concurrently, they live with the constant threat of unpredictable incidents of hypoglycemia and the persistent worry of future damage associated with the disease state. Therefore, these patients are probably not enthusiastic about the prospect of waiting for the day in the distant future when the resolution of all the problems of the extremely interesting, yet extremely complex process of tissue regeneration are resolved, before they can have a cure. They would likely look forward to a cure sooner, rather than later. However, it is only in the movies where optimism always prevails against all odds. In science, optimism is generally tempered by the concerns and critiques of peers, which serve to raise questions and may actually rectify errors. Despite some scientific skepticism, the prospect of gene therapy-based treatments remains intriguing and the use of human stem cell research carries with it enormous scientific potential in the treatment and possible cure of many diseases. As we wait, then, for a successful and perhaps not too distant clinical application of the regenerative capabilities of our endocrine pancreas, we may have to be hopeful and just satisfied by the obtained evidence that supports the rights for the beta cell to proudly repeat the statement made famous by the T-800:

'I'll be back!'.

Acknowledgements

I would like to thank my son, Matteo, for introducing me to Cameron's movies; and Sharon Rosenshine, who always helped me to improve my written English.

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Benchmarks

An improved intracellular staining protocol for efficient detection of nuclear proteins in YFP-expressing cells

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BioTechniques 51:417-420 (December 2011) doi 10.2144/000113780
Keywords: Intracellular staining; Flow Cytometry; Membrane Permeabilization; YFP; Foxp3; and T regulatory cells.

Supplementary material for this article is available at www.BioTechniques.com/article/113780.

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Intracellular staining is a widely used flow cytometry (FCM)-based technique to detect the expression of cytosolic or nucleic antigens. However, intracellular staining of cells expressing cytosolic fluorescent protein (FP) markers was proven to be problematic as significant loss of the FP-signal was routinely observed. Using splenocytes harvested from mice constitutively expressing the enhanced yellow fluorescent proteins (YFP) as a model, we modified the widely used intracellular staining protocol and successfully achieved simultaneous detection of both the nuclear proteins and YFP in T-regulatory cells. The improved protocol can be used to perform antibody-based intracellular characterization of FP-labeled target cells, while maintaining their fluorescent reporter signals for easy tracing and identification.

Transgenic animals expressing a fluorescent protein (FP) in specific cell types or lineages are essential tools for studying biological processes in vivo (1). Frequently, to target a specific cell population, the promoter element governing the transcription of a tissue-specific gene is used to control FP expression directly or indirectly through the action of the Cre recombinase in a Rosa26-FP reporter line (2). Cre-mediated removal of the loxP-tagged transcriptional stopper cassette situated between the ubiquitously active Rosa26 promoter and the FP gene will switch on the FP gene transcription, resulting in permanent labeling of cells of the specific lineage with FP(3,4).

Nevertheless, it is essential to examine whether the promoter elements used to drive FP expression can truthfully recapitulate the tissue specificity of the endogenous gene in vivo (4). Staining FP-positive cells with antibodies specific to the endogenous proteins for flow cytometry (FCM) analysis is an effective way to validate the nature of the FP-labeled cells (5). In addition, antibody-based FCM can also facilitate the characterization of the molecular properties of the FP-labeled cells under various experimental conditions (6). However, FCM detection of cytosolic or nuclear proteins in FP-expressing cells has been shown to be technically challenging as loss of FP signal is frequently observed when the intracellular staining procedure is used. The common approach to overcome this obstacle is to first isolate the FP-expressing cells with the fluorescence activated cell sorter (FACS) technique, followed by intracellular characterization (7). Such an approach is not only time-consuming, but also impractical when studying rare cells.

In this study, we modified an intracellular staining protocol, which utilizes paraformaldehyde and saponin as the fixative and membrane permeabilizating reagent, respectively, to achieve simultaneous detection of nuclear proteins and cytosolic FP molecules. The nuclear Foxp3 proteins in T-regulatory cells of Vav-Cre:Rosa-YFP reporter mice (3) were used as a model target.

After staining YFP+ splenocytes with antibodies specific to surface markers of T cells (i.e., CD3 and CD4), we subjected them to the intracellular staining procedure outlined in Figure 1A (detailed in Supplementary material) for the detection of nuclear Foxp3 proteins. Although the Foxp3+ T-regulatory cell population could easily be identified, the YFP signals in these cells became essentially undetectable (Figure 1B). Loss of YFP signal could result either from fixative-induced conformation changes of the YFP proteins, or from the loss of cytosolic YFP proteins due to the leakiness of the permeabilized cell membrane. To investigate whether overfixation is the major causative factor, we shortened the Fixation/Permeabilization (Fix/Perm) buffer treatment from 2 hours to 5 minutes, but still failed to preserve detectable YFP signals (data not shown). Indeed, significant reduction of cellular YFP signal was observed immediately following exposure of YFP+ cells to the Fix/Perm buffer, indicating that the rapid loss of the fluorescent signal is due to diffusion of YFP molecules out of the permeabilized cells (Figure 1C and data not shown).

As fixation with 1%–2% paraformaldehyde is routinely used in FCM protocols to preserve fluorescent signals for analysis, we postulated that adding a fixation step prior to the Fix/Perm treatment might prevent YFP proteins from leaking out of the cytoplasmic membrane. Indeed, prefixing samples with 2% paraformaldehyde for 30

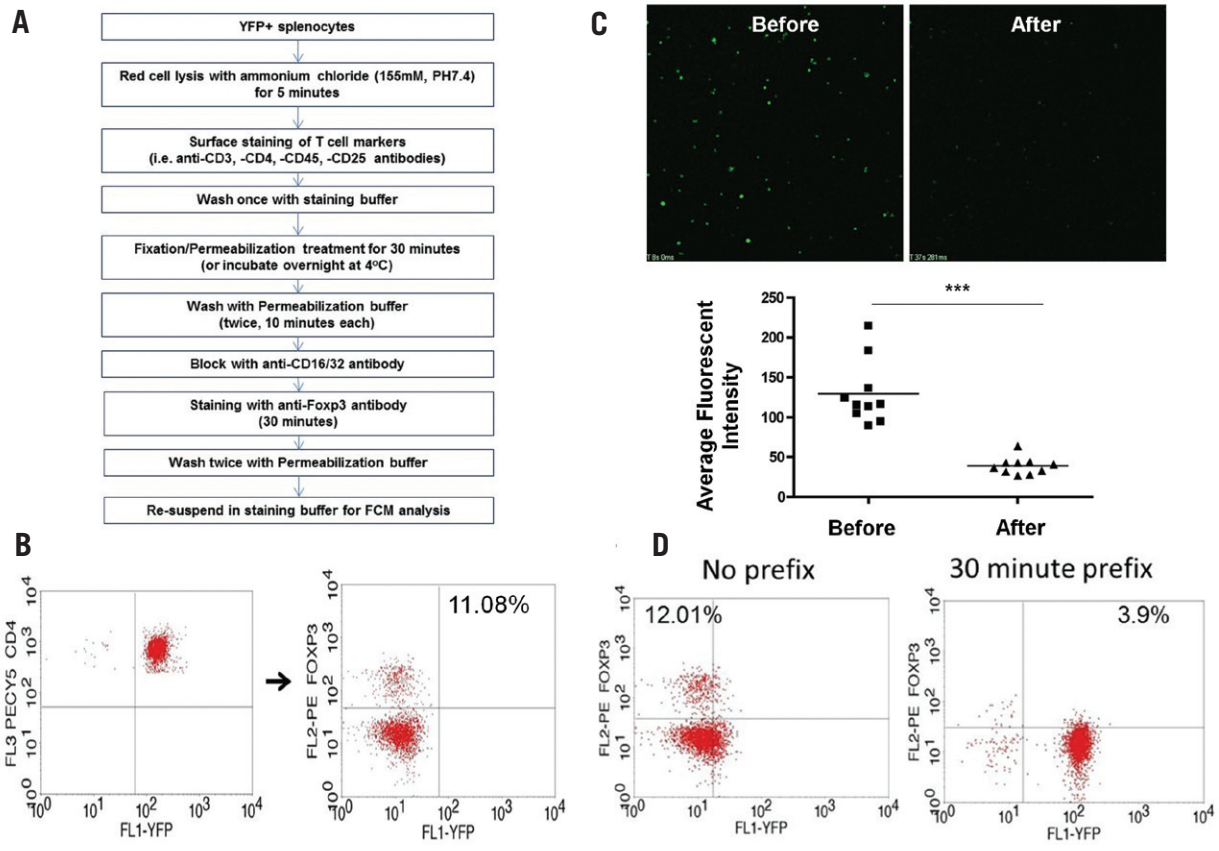

Figure 1. Loss of YFP signal in cells treated with Fixative/Permeabilization reagents used in intracellular staining procedures. (A) A step-by-step protocol for intracellular staining of nuclear Foxp3 protein in T regulatory cells. (B) YFP+, CD4+T cells harvested from Vav-Cre:Rosa-YFP mice were stained intracellularly with anti-Foxp3 antibodies following the procedure outlined in (A). Left panel, before Foxp3 staining; Right panel, after Foxp3 staining. (C) Representative fluorescent microscopy images of YFP+ splenocytes, showing rapid loss of YFP signal in YFP+ cells upon Fix/Perm exposure. Top panels: Left, prior to Fix/Perm exposure; Right, 5 s after Fix/Perm addition to the medium. Lower panel: Average fluorescent intensities of YFP+ cells (n = 10) before or after of Fix/Perm buffer addition. ***, $P < 0.0001$ (Student's t test). (D) Intracellular staining of Foxp3 is significantly compromised in YFP+ splenocytes prefixed with 2% paraformaldehyde for 30 min, while YFP signal is well-preserved. Shown are representative FCM results from three independent experiments.

minutes can effectively retain the YFP signal; however, we then became unable to stain Foxp3 proteins (Figure 1D), presumably due to overfixation of the cytoplasmic membrane. We thus speculated that the success of staining the nuclear FoxP3 molecules, while preserving detectable YFP signals, might hinge on the optimization of pre-fixation of cytoplasmic membrane: to block the leakage of YFP molecules from cytosol, while preserving the accessibility of the antibody to its nuclear target.

To find out the minimal fixative conditions which can be used to preserve the YFP signal, cells were prefixed with various concentrations of fixative for different lengths of time (Supplementary Table 1), followed by Fix/Perm treatment and FCM analysis. As summarized in Figure 2A, the success of cytoplasmic YFP protein retention was determined by the combined effect of prefixation time and concentration of the fixative. To give a relative quantification of the prefixation process, we defined the value of prefixation time (minutes) x % of fixative as prefixation factor (PF), and concluded that conditions with PF≥4 (gray area in Figure 2A) were sufficient to retain enough YFP proteins for FCM analysis.

To evaluate the negative effect of prefixation on Foxp3 staining, we took advantage of the eGFP-Foxp3 transgenic mice, in which an eGFP molecule was inserted in-frame to the 5′ end of the *foxp3* gene to encode a functional eGFP-Foxp3 fusion protein (8). One of the major functional components in the permeabilization buffer is saponin, which can complex with cholesterol to form pores in the cholesterol-rich cytoplasm membrane but leaves the cholesterol-poor nuclear membrane largely intact (9,10). Thus, nuclear eGFP-Foxp3 fusion protein should be retained in the nucleus during intracellular staining while the eGFP signal will truthfully reflect the presence of Foxp3 protein (Figure 2B). Splenocytes harvested from the eGFP-Foxp3 transgenic mice were prefixed with 2% paraformaldehyde from 15 seconds to 5 minutes, prior to staining of the cells with anti-Foxp3 antibodies. Prefixation from 15 seconds to 2 minutes did not drastically affect the percentage of Foxp3+ cells, whereas we began to observe a decrease of the antibody staining of the eGFP-Foxp3 fusion proteins after the cells were treated with fixative for more than 5 min (Figure 2B and data not shown).

Based on the above findings, we proceeded to add the prefixation step to the intracellular staining protocol for Foxp3 detection in Vav-Cre: RosaYFP T regulatory cells. As shown in Figure 2C, a distinct population of CD4+ T cells can

be readily identified as double positive for both YFP and Foxp3 under a number of amenable conditions. Since various intracellular antigens may differ substantially in their retention and susceptibility to Fix/Perm treatment, we also examined the nuclear expression of *helios*, a member of the Ikaros family of nuclear expressed transcription factors, in YFP+ spleno-

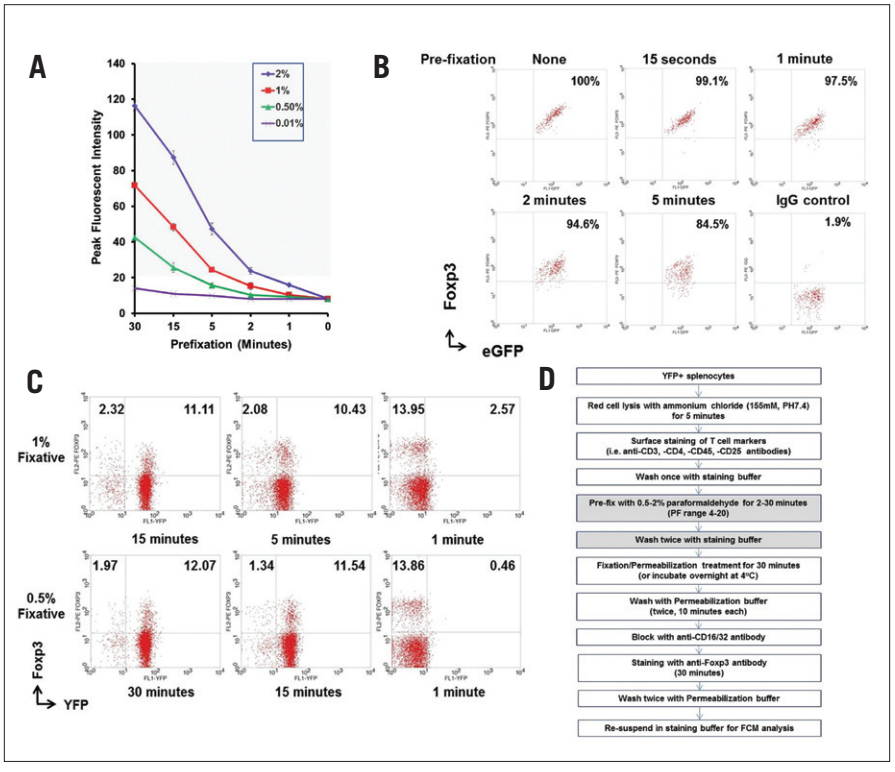

Figure 2. Short Prefixation prior to intracellular staining is sufficient to retain detectable cytosolic YFP signals. (A) Systematic evaluation of YFP signal preservation in YFP+ splenocytes treated with various prefixing conditions, followed by Fix/Perm exposure. Gray area: conditions under which sufficient YFP signals were preserved for FCM detection. Percentages (%) of paraformaldehyde used as fixative are shown in the insert. The results are summarized from four independent experimental repeats. (B) Splenocytes harvested from eGFP-Foxp3 mice were pretreated with 2% paraformaldehyde for various times (shown on the top of each panel), and subsequently stained intracellularly with anti-Foxp3 antibodies and analyzed by FCM (gated on CD4+eGFP+ T regulatory cell population). Percentages of cells with nuclear Foxp3 stained successfully under different pre-fixation conditions are shown. These results are representative of FCM analyses from five independent experiments. (C) Foxp3-staining of Vav-Cre:RosaYFP CD4+ T cells pre-treated with either 0.5% or 1% of paraformaldehyde for the times shown below each panel. Percentages of CD4+ T cells positive for both Foxp3 and YFP are shown on the upper right corners. Shown are representative results from five independent experiments. (D) Modified intracellular staining protocol for nuclear Foxp3 detection. Steps in gray boxes were added to the original procedure in Figure 1A. For other cytosolic or nuclear proteins, optimization of the pre-fixation time may be required.

cytes (11). As shown in Supplementary Figure S1, effective detection of both Helios and YFP were achieved under conditions similar to those described in Figure 2C. Thus, PFs range from 4 to 20 represents the optimal conditions to obtain efficient nuclear protein staining, and well separation of the YFP signals from the background. It is conceivable that the optimal PF will vary in different models; for cells with weak fluorescent signals, a higher PF (10–20) could be preferred, and vice versa. Nevertheless, the simple modification of the intracellular staining procedure described above (Figure 2D) reliably enables the usage of cytoplasmic FPs as cellular markers for co-localization studies with FCM technology.

Acknowledgments

We thank Dr. Joy Williams for helpful discussions and critical reading of the manuscript. We also thank Mr. Carl Engman, Dr. Brett Philips and Dr. Valentina DiCaro for technical assistance. The study was supported in part by Department of Defense grant W81XWH-09-1-0742.

Competing interests

The authors declare no competing interests.

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Received 8 September 2011; accepted 12 October 2011.

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Cellular Immunology 273 (2012) 115–123

Contents lists available at SciVerse ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Essential roles of insulin expression in Aire$^+$ tolerogenic dendritic cells in maintaining peripheral self-tolerance of islet β-cells ☆

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ARTICLE INFO

Article history:
Received 28 October 2011
Accepted 27 December 2011
Available online 12 January 2012

Keywords:
Insulin
Tolerogenic dendritic cells
Peripheral tolerance
Autoimmune diabetes

ABSTRACT

Anti-insulin autoimmunity is one of the primary forces in initiating and progressing β-cell destruction in type 1 diabetes. While insulin expression in thymic medullary epithelial cells has been shown to be essential for establishing β-cell central tolerance, the function of insulin expression in antigen-presenting cells (APCs) of hematopoietic lineage remains elusive. With a Cre-lox reporter approach, we labeled Aire-expressing cells with enhanced yellow fluorescent proteins, and found that insulin expression in the spleen was restricted predominantly to a population of Aire$^+$CD11c^{int}B220$^+$ dendritic cells (DCs). Targeted insulin deletion in APCs failed to induce anti-islet autoimmunity in B6 mice. In contrast, elevated levels of T cell infiltration into islets were observed in B6^{g7} congenic mice when insulin was specifically deleted in their CD11c-expressing DCs (B6^{g7}.CD11c-ΔIns mice). Thus, insulin expression in BM-derived, Aire$^+$ tolerogenic DCs may play an essential role to prevent the activation and expansion of insulin-reactive T cells in the periphery.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-secreting pancreatic β-cells are selectively destroyed by the patients' own immunocompetent cells. Although numerous islet autoantigens, such as glutamate decarboxylase 65 (GAD65), insulinoma-associated protein (IA)-2, islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP), islet cell autoantigen 69 (ICA69), and zinc transporter (Znt) 8, have been identified in T1D etiology [1–6], anti-insulin autoimmunity remains as perhaps the most important driving force for the initiation and progression of the disease [7–9]. Autoantibodies specific to insulin can be detected in patients years before the clinical onset of diabetes, and their presence has been used as one of the key biomarkers (together with anti-GAD65 and IA-2 autoantibodies) to predict diabetes onset in genetically high risk individuals [10–12]. T-cells with TCRs specific to insulin epitopes have been identified as the predominant population of islet infiltrating T-cells in prediabetic nonobese diabetes (NOD) mice [13]. Moreover, it was shown recently that strengthening peripheral insulin immune tolerance through immunizing prediabetic NOD mice with a dominant insulin mimetope can effectively prevent diabetes onset and anti-islet autoimmunity progression, further implicating the pivotal roles of anti-insulin autoimmunity in islet destruction [14].

Indeed, the second highest genetically susceptible locus (IDDM2) in human T1D was located to a region proximal to the promoter of the human insulin gene, which contains a variable number of tandem nucleotide repeats (VNTR) [15,16]. The length of the VNTR region influences the level of thymic insulin expression: individuals carrying shorter alleles have lower numbers of insulin transcripts and are more susceptible to T1D, whereas those with longer VNTR have higher numbers of insulin transcripts and are more resistant to the disease [17,18]. Similar reverse correlation between the thymic insulin levels and the degrees of anti-insulin autoimmunity in the periphery was also observed in animal models [19]. Indeed, significant acceleration of diabetes onset was observed in NOD mice with abrogated *Ins2* gene expression [20]. To further investigate the roles of thymic insulin expression in establishing β-cells tolerance, we have recently generated the ID-TEC mouse model (for insulin deletion

Abbreviations: FACS, fluorescent activated cell sorting; FCM, flow cytometry; CRE, bacteriophage P1 Cre recombinase; DC, dendritic cell; APC, antigen presenting cell; EYFP, enhanced yellow fluorescent protein; BM, bone marrow.

☆ This study was supported in part by Department of Defense Grant # W81XWH-09-1-0742 and the Ri.Med Foundation.

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doi:10.1016/j.cellimm.2011.12.010

in thymic epithelial cells), in which insulin expression is specifically abrogated in Aire-expressing mTECs, whereas its production in the pancreas remains intact [21]. Both male and female ID-TEC pups developed autoimmune diabetes spontaneously 3 weeks after birth, due to destruction of islet β-cells. In contrast, exocrine and other endocrine cells such as glucagon-secreting α-cells were largely spared. Thus, insulin expression in medullary TECs is essential for the negative selection of insulin-reactive T cells and to establish a T cell repertoire tolerant to pancreatic β-cells.

Notably, insulin expression was also reported in BM-derived APCs [22–24], although discordant results have been reported regarding the specific cell subsets. Both (pro)insulin transcripts and proteins were detected in human $CD11c^{+}$ dendritic cells (DCs) in the thymus and peripheral lymphoid tissues [23–24]. In contrast, Hansenne et al. found neither *Ins1* nor *Ins2* transcripts in mouse $CD11c^{high}$ DCs, regardless of their maturation status [25]. Nevertheless, transplantation of BM cells harvested from NOD.$Ins2^{+/+}$ mice failed to slow down diabetes progression in NOD.$Ins2^{-/-}$ recipients, suggesting that endogenous levels of *Ins2* expression in BM-derived cells of NOD mice cannot restore peripheral tolerance to insulin [26]. However, the failure could be attributed to the low levels of *Ins2*-expression in the transplanted BM cells, as it was shown that the levels of *Ins2*-expression in spleen and pancreatic lymph nodes (LN) decrease significantly after weaning (3–4 weeks) in NOD mice [27]. Thus, the role of insulin expression in secondary lymphoid tissues in regulating peripheral tolerance of β-cells remains elusive.

In this study, we generated animal models to better investigate the immunomodulatory function of insulin expression in BM-derived cells and establish their role in maintaining peripheral immune tolerance to pancreatic β-cells.

2. Materials and methods

2.1. Animals

Both the *Ins2* floxed (*Ins2*$^{flox/flox}$) and the *Ins1*-knockout (*Ins1*$^{del/del}$) mice are on the C57BL/6 background and have been described previously [21]. Vav-Cre transgenic mice were obtained through pronuclear injection of a linearized Vav1-Cre construct into fertilized eggs harvested from B6; 129 F1 mice and were subsequently crossed to the C57BL/6 background. CD11c-Cre mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Both transgenic lines were bred with *Ins1*$^{del/del}$:*Ins2*$^{flox/flox}$ mice to obtain the Vav-ΔIns (Vav-Cre:*Ins1*$^{del/del}$:*Ins2*$^{flox/flox}$) and the CD11c-ΔIns (CD11c-Cre:*Ins1*$^{del/del}$:*Ins2*$^{flox/flox}$) mice, respectively. All the animals mentioned above are on the C57BL/6 genetic background with the MHC H-2^{b} haplotype. To obtain the B6^{g7}·CD11c-ΔIns mice, CD11c-ΔIns mice were crossed to congenic B6.H-2^{g7} mice (Jackson Laboratory) to fix the H-2^{g7} MHC alleles.

The Aire-Cre mouse was described previously [21]. Vav-Cre:Rosa26R-EYFP and Aire-Cre:Rosa26R-EYFP mice were generated via crossing female Rosa26R-EYFP (Jackson laboratory) to male Vav-Cre and Aire-Cre mice, respectively. All mice were housed in a specific pathogen-free animal facility at Rangos Research Center, Pittsburgh. All animal experiments were carried out under protocols approved by the Institutional Animal Care and Usage Committee of the University of Pittsburgh.

2.2. Genotyping of genetically modified animals

0.5 cm tail biopsies were surgically removed from 14-20 day old pups. Genomic DNA was isolated with DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). Primer pairs used in the study were:

Gene	Primers	Genotyping
Cre recombinase	5′-GAGTGATGAGGTT CGCAAGAA-3′ 5′ GCGCGCCTGAAG ATATAGAAG 3′	Annealing temperature: 60 °C. Cre PCR product: 150 base pairs
Ins2	5′-GACTCTGGGAAAG GCATCTGTTCAG-3′ 5′-TTGCTTACAGTCTA TTTTCAGGGTC-3′	Annealing temperature: 65 °C. Ins2 floxed allele: 225 base pairs. Ins2 wild type allele: 154 base pairs
Ins1	5′-CCAGATACTTGAATT ATTCCTGGTGTTTTAT CAC-3′ 5′-GCT GCA CCA GCA TCT GCT CCC TCT ACC-3′ 5′-TTC TCG GCA GGA GCA AGG TGA GAT GAC-3′	Annealing temperature: 65 °C. Ins1 KO allele: 550 base pairs. Ins1 wild type allele: 273 base pairs

H-2 haplotypes were determined by staining PBMCs with anti-MHC class I antibodies specific for H-$2K^{b}$ and H-$2K^{d}$, clones AF6-88.5 and SF1-1.1, respectively (BD Biosciences, San Diego, CA).

2.3. Blood glucose and Intraperitoneal glucose tolerance test (IPGTT) and hematocrit measurement

Blood glucose levels were measured with the Ascensia Contour blood glucose monitoring system (Bayer HealthCare LLC, Mishawaka, IN). To perform IPGTT, mice were fasted overnight (~16 h) and injected intraperitoneally with 2 g of D-glucose (Sigma-Aldrich, St. Louis, USA) per kilogram of body weight. Blood was sampled from a small nick of the tail-vein at 0, 15, 30, 60, 90 and 120 min after glucose injection. Direct hematocrit measurements were determined on fresh whole blood samples harvested from 40-week old females by Heska I-Stat Portable Clinical Analyzer (Heska, Loveland, CO).

2.4. RNA analysis

The total RNA of spleens or pooled thymi was isolated using an RNA minikit, according to the manufacturer's protocol (Qiagen). Following DNase I treatment (Ambion), RNA samples were reverse-transcribed into cDNAs with Superscript III cDNA kit, (Invitrogen). qPCR analyses of gene expression in cDNA samples were performed with the LightCycler FastStart DNA Master SYBR Green I kit, and analyzed with the LightCycler 2 software (Roche Applied Science). Unless specified, annealing temperatures for all PCR reactions were set at 60 °C. Primer pairs used in the study:

Hprt	F 5′-GGATACAGGCCAGACTTTGTTGGA-3′ R 5′-CAACAGGACTCCTCGTATTTGCAG-3′
Gr-1	F 5′-TCAAGAGCAATCTCTGCCTTCCCA-3′ R 5′-AGGACTGAAACCAGGCTGAACAGA-3′
NK1.1	F 5′-TGCCAGACATGAACTGGAAGTGGA-3′ R 5′-TGGCAGATCCAACGGTTGTCTGAA-3′
Insulin	F 5′-CACCCAGGCTTTTGTCAA-3′ R 5′-AGATGCTGGTGCAGCACT-3′
β-actin	F 5′-TGGAATCCTGTGGCATCCATGAAA-3′ R 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

2.5. Histology and Immunohistochemistry

Pancreata, spleens and thymi were harvested, fixed in 4% paraformaldehyde for 3 h at 4 °C, and placed in 30% sucrose overnight. Cryosections of 5 μm thick were cut and stained with primary antibodies. Antibodies used in the study: B220, CD45, CD4 and CD8 (BD Biosciences, Franklin Lakes, NJ); Insulin (Santa Cruz Biotechnology, Santa Cruz, CA); Glucagon (Zymed, San Francisco, CA). To quantify percentages of islets infiltrated by lymphocytes, 5–6 pancreata were harvested and analyzed from either $B6^{g7}$.CD11c-ΔIns or control $B6^{g7}$ mice. At least 10 islets per mouse were examined by two observers unaware of the sample origins.

2.6. Flow cytometry

Flow cytometric analysis was performed on the BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed with CellQuest Pro software (BD Biosciences). Single cell suspensions were prepared from spleen, subjected to erythrocyte depletion in red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO), blocked with anti-CD16/32 antibody and stained with antibodies. The following antibodies were purchased from BD Biosciences: anti-CD16/32 (2.4G2), anti-CD4 PeCy5 (H129.9), anti-CD45-APC (30-F11), and anti-CD3-APC (145-2C11). Anti-CD25-APC (7D4) antibody was purchased from Miltenyi Biotec (Auburn, CA). Staining buffer: phosphate buffered saline (PBS, calcium and magnesium free, Invitrogen) supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich).

Intracellular staining of the Foxp3 protein was performed with commercial kit purchased from eBiosciences (San Diego, CA), following manufacturer's suggested protocol.

2.7. Isolation of stromal cells from lymphoid organs

Spleens harvested from Aire-Cre:Rosa26R-EYFP mice were dissected into 1 mm pieces, and digested with collagenase D (Roche) solution into single cells as previously described [19]. After treatment with red cell lysis buffer (Sigma-Aldrich), single cells were subjected to magnetic bead isolation to positively select $CD45^+$ bone marrow derived cells, following manufacturer's protocols (Miltenyi Biotec, $CD45^+$ cell isolation kit). Cells harvested were stained with anti-CD45, anti-B220, anti-CD11c, anti-IA/IE, and anti-NK1.1 antibodies and analyzed with FACSCalibur as described above. To enrich $EYFP^+$ cells, isolated splenocytes were stained with anti-CD11c and anti-CD45 antibodies, and sorted by FACS. Purities above 95% were achieved routinely.

2.8. ELISPOT assay

ELISPOT assays were performed using the BD mouse IFN-γ ELIPOT set, according to the manufacturer's specifications (BD Biosciences). Splenocytes (3×10^6) from two to three mice were pooled and cultured overnight with 0.1 mg/ml insulin (Sigma-Aldrich), whole GAD65 protein (Abnova), insulin B chain peptide 9-23 (SHLVEALYLVCGERG, AnaSpec), GAD65 peptide 206-220 (TYEIAPVFVLLEYVT, Sigma-Aldrich) or medium alone. Assays were repeated and performed in triplicate and averaged.

2.9. Statistical analysis

Statistical significance was determined using paired, Student *t*-test. In all experiments, differences were considered significant when p was less than 0.05.

3. Results

3.1. Tissue-specific deletion of the mouse Ins2 gene in BM-derived APCs

To investigate the immunomodulatory role of insulin expression in BM-derived cells, we took advantage of the pan-hematopoietic expressing feature of the *Vav1* gene during early embryogenesis and generated the Vav-Cre:$Ins1^{del/del}$:$Ins2^{flox/flox}$ line (designated as Vav-ΔIns mice) to abrogate insulin expression in cells of hematopoietic lineages [28,29]. As a first step, we validated the efficiency of the Vav-Cre transgene in mediating floxed-gene deletion by crossing the Vav-Cre transgenic mouse to a Rosa26R-EYFP reporter line. Cre-mediated recombination enables the expression of EYFP from the ubiquitously active Rosa26 promoter. Consistent with previous reports, peripheral blood mononuclear cells harvested from the Vav-Cre:Rosa26R-EYFP mice are almost all EYFP positive (Supplementary Fig. 1A) [28–29]. Furthermore, efficient recombination was detected in splenocytes of all hematopoietic lineages, including T cells, B cells, macrophages, DCs, and NK cells (Supplementary Fig. 1B). Of particular note, Vav-Cre mediated EYFP expression was not detected in insulin secreting β-cells of the pancreas (data not shown).

As ectopic insulin expression has been reported in BM-derived APCs within the thymus [23,24,30], we also examined EYFP expression in thymic APCs. As shown in Fig. 1A, >95% of MHC II^+ thymic DCs, macrophages and B cells were EYFP positive. In contrast, <15% of $CD45^-EpCAM^+$ thymic epithelial cells (including both cortical and medullary epithelial cells) were EYFP-positive. These results indicate that the Vav-Cre transgene can efficiently delete the floxed genes in thymic APCs of hematopoietic origin, but has only limited impact on mTECs (Fig. 1A).

To validate that *Ins2* gene was specifically deleted in BM-derived APCs, Southern blot analysis was performed on genomic DNA harvested from BM cells of Vav-ΔIns mice. As shown in Fig. 1B, the floxed *Ins2* band is essentially undetectable, indicating near total deletion of the *Ins2* gene in hematopoietic lineages. Of particular note, thymic insulin expression is largely unaffected in Vav-ΔIns mice (Fig. 1C and 1D), consistently with our and others' previous findings that insulin transcripts are predominantly present in thymic mTECs [19,21,31].

3.2. Absence of anti-insulin autoimmunity in Vav-ΔIns mice

Vav-ΔIns mice were born healthy without any obvious physiologic abnormalities, nor did they display any defect in maintaining blood glucose homeostasis throughout their lifespan (Fig. 2A and data not shown). Compared with littermate controls, Vav-ΔIns mice responded normally to a glucose challenge, suggesting that there are no abnormalities in glucose metabolism and islet function (Fig. 2B). Histologically, normal islet structure and insulin-producing β-cells were observed in pancreata harvested from Vav-ΔIns mice. Neither damaged islets nor lymphocyte infiltration (i.e., insulitis) were observed, suggesting that there was no ongoing anti-islet autoimmunity within the pancreas (Fig. 2C). Furthermore, only background levels of T cell responses to insulin were observed in ELISPOT assays of Vav-ΔIns splenocytes, indicating that there was no change in the abundance of insulin-specific autoreactive T cells in the periphery (data not shown). Notably, the Vav-ΔIns mice are in C57BL/6 background with autoimmune diabetes resistant MHCs ($H\text{-}2^b$ haplotype), and their ectopic insulin expression in mTECs is largely intact. These data indicate that elimination of insulin expression in BM-derived thymic APCs alone will neither impair the central negative selection of insulin-reactive T-cells, nor cause breakdown of immune tolerance to pancreatic beta cells in the periphery.

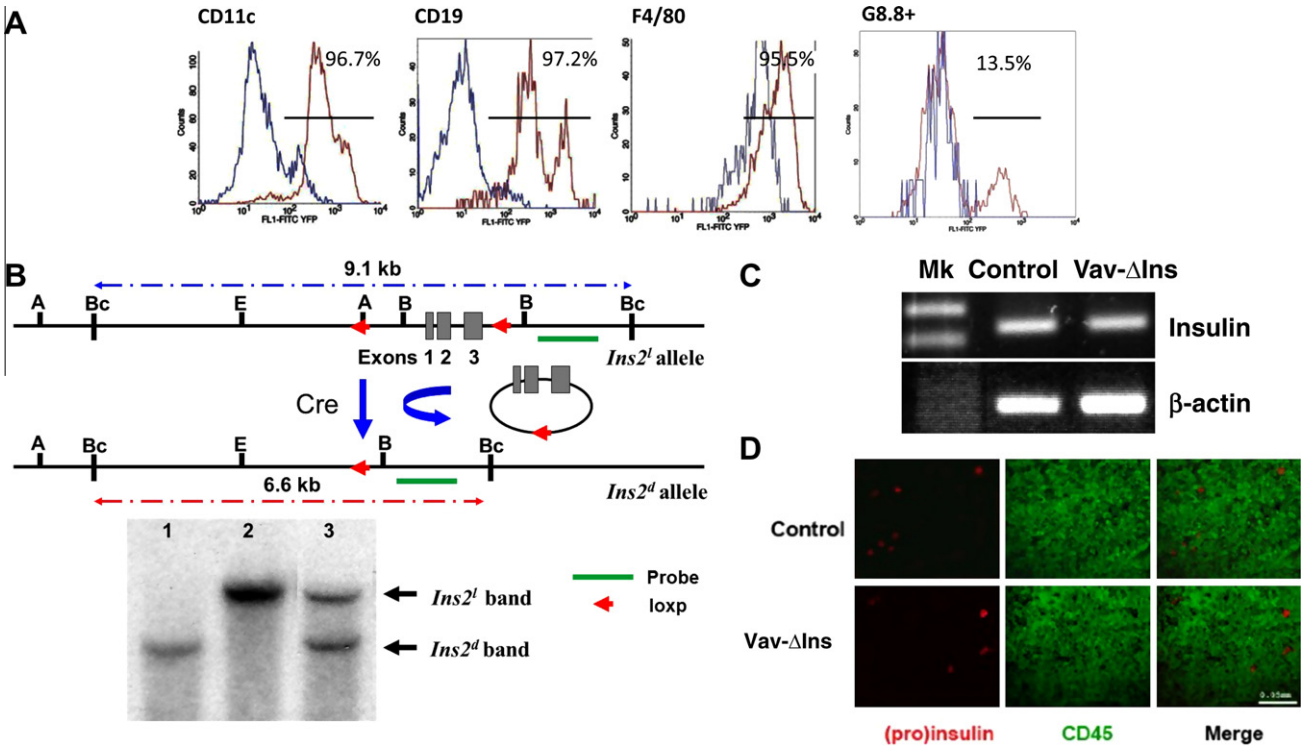

Fig. 1. Deletion of the mouse insulin gene in hematopoietic lineage derived cells. (A) Stromal cells harvested from the thymi of Vav-Cre:Rosa26R-EYFP mice were depleted with anti-CD90 magnetic beads and separated with hematopoietic lineage marker CD45 magnetic beads. $CD45^{+}MHC\ II^{+}$ cells were stained and gated on the specific lineage markers shown. $CD45^{-}$ cells were gated on epithelial cell marker $g8.8^{+}$ (EpCAM). Histograms with red outlines represent results from Vav-Cre:Rosa26R-EYFP mice, whereas histograms with blue lines represent Rosa26R-EYFP controls. Shown is a representative FCM result from three independent experiments of 2-3 animals each. (B) *Top panel*, schematic drawing of Cre-mediated deletion of the floxed Ins2 allele. Gray boxed marked 1-3 are Ins2 exons. The distances between the two restrictive endonuclease enzyme BclI recognition sites for the floxed Ins2 allele ($Ins2^{l}$) and the Ins2 deletion allele ($Ins2^{d}$) are 9.1 kb (blue line) and 6.6 kb (red line), respectively. A, AseI; B, BamHI; Bc, BclI; E, EcoRI. *Lower panel*, Genomic DNA harvested from bone marrow cells were digested with endonuclease BclI, separated by electrophoresis and transferred to the nylon membrane for Southern blot analysis. *Lane 1*, Vav-ΔIns mice; *lane 2*, homozygous $Ins2^{flox/flox}$ mice; *lane 3*, heterozygous $Ins2^{flox/del}$ mice. C. RT-PCR analysis of thymic insulin expression in Vav-ΔIns mice. D. Immunohistochemical analysis of thymi harvested from Vav-ΔIns mice and controls. Cryosections were labeled with anti-(pro)insulin (red) and anti-CD45 (green) antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

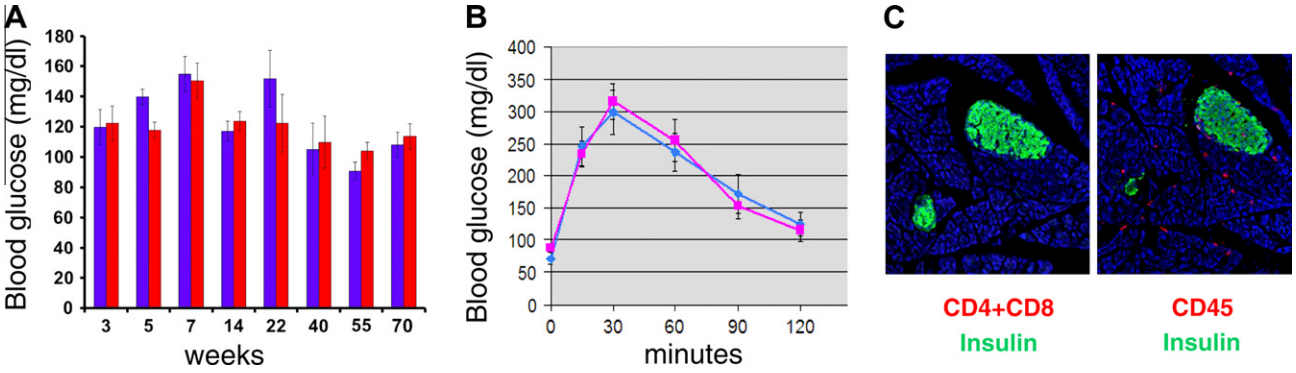

Fig. 2. Absence of anti-insulin autoimmunity in Vav-ΔIns mice. (A) Blood glucose levels of Vav-ΔIns mice (red bar, $n = 8$) and littermate controls (blue bar, $n = 8$). (B) Intraperitoneal glucose tolerance test (IPGTT). No difference was observed in Vav-ΔIns (red line, $n = 8$) and control littermates (blue line, $n = 8$). (C) Representative sections of immunohistochemical analysis of pancreata harvested from Vav-ΔIns mice and controls. Left panel, cryosections were labeled with anti-(pro)insulin (green), together with anti-CD4 and anti-CD8 (red) antibodies. Right panel, adjacent section was labeled with anti-insulin and anti-CD45 antibodies, showing the presence of BM-derived cells in the pancreas. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Ectopic insulin expression in BM-derived, Aire-expressing tolerogenic APCs in the spleen

We then began to identify the subset(s) of BM-derived APCs which express insulin. Since thymic *Ins2* expression was shown to be regulated by the autoimmune regulator (Aire) gene, and that low levels of Aire gene expression were reported in BM-derived $CD11c^{+}$ dendritic cells (DCs) [21,32–34], we examined whether insulin is also expressed in Aire-expressing cells of BM-origin. Taking advantage of our previous findings that the Aire-Cre transgene can faithfully recapitulate the endogenous Aire gene expression pattern [21], we crossed the Aire-Cre transgenic mice to Rosa26R-EYFP mice to label Aire-expressing cells with EYFP. FCM analyses showed that Aire-expressing cells ($EYFP^{+}$) represented about 1–2% of the $CD45^{+}$ population in the spleen (Fig. 3A). They expressed low to intermediate levels of the DC marker CD11c, as

well as high levels of MHC II and B220 molecules (Fig. 3B). Of note, similar molecular markers (CD11c^{low}MHC II^{+}B220^{+}) were found on two recently described tolerogenic DC subsets, the plasmacytoid DCs (pDCs) [34] and the interferon-γ (IFN-γ)-secreting natural killer DCs (NKDCs) [35].

To further characterize the Aire-expressing cells, we separated the CD11c^{+} cells into three populations: the CD11c^{high}EYFP^{-} "conventional" DCs (P1), the CD11c^{int}EYFP^{-} DCs (P2) and the CD11c^{int}EYFP^{+} "tolerogenic" DCs (P3), and subjected them to RT-qPCR analyses of genes expressed in specific DC subsets. It was previously shown that Gr-1 is highly expressed in pDCs, whereas the NK cell marker NK1.1 is predominantly found in NKDCs [36–38]. As illustrated in Fig. 3C, cells in the CD11c^{int}EYFP^{-} P2 population expressed higher levels of NK1.1 but lower levels of Gr-1, suggesting that the P2 population was comprised primarily of NKDCs. In contrast, high levels of Gr-1 transcripts were found in the CD11c^{int}EYFP^{+} P3 population, whereas the NK1.1 signal was barely detectable, suggesting that these cells expressed markers similar to pDCs (Fig. 3C). However, further analyses of surface markers revealed that only about 5% of the P3 population expressed the

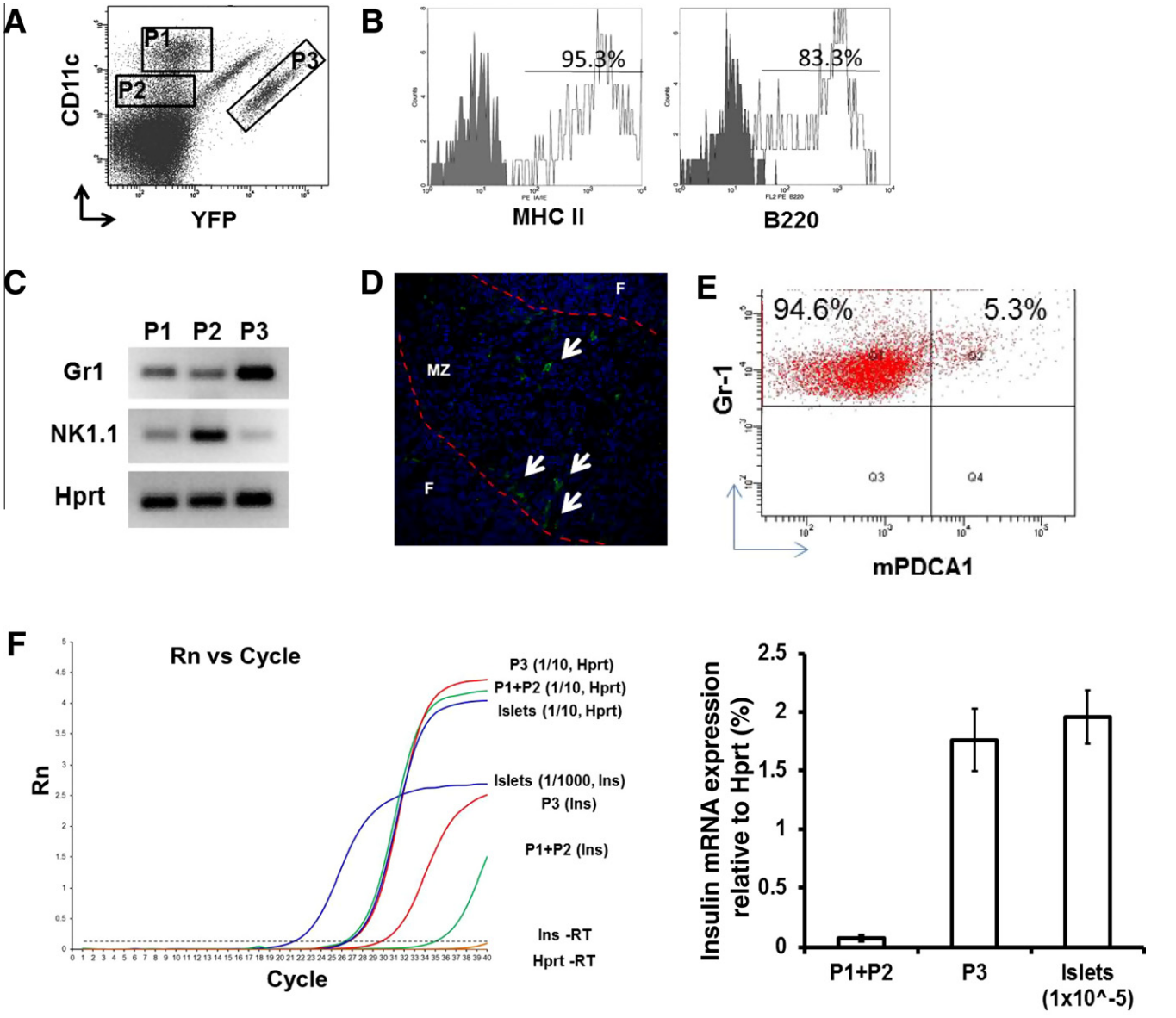

Fig. 3. Insulin expression in the spleen is largely restricted to Aire^{+}CD11c^{int}B220^{+} tolerogenic pDCs. (A) FCM analysis of splenocytes isolated from Aire-Cre:Rosa26R-EYFP mice. Cells were positively selected by anti-CD45 conjugated magnetic beads, followed by staining with anti-CD45 and anti-CD11c antibodies. As shown, CD11c^{+} cells (gated on CD45^{+}) were separated into three populations: P1, CD11c^{high}EYFP^{-}; P2, CD11c^{int}EYFP^{-}; P3, CD11c^{int}EYFP^{+}. Shown is a representative FACS result from three independent experiments of spleens pooled from three animals. (B) Cells of P3 population was sorted and stained with anti-MHC II and anti-B220 antibodies (solid lines), or IgG controls (filled gray lines). (C) RT-PCR analysis of expression of genes specific to subsets of DCs. Gr1, pDCs; NK1.1, NKDCs. (D) Immunohistochemical analysis of spleen sections of Aire-Cre:Rosa26R-EYFP mice. 5 μm cryosections were probed with anti-EYFP antibody (green). As shown, EYFP^{+} cells are predominantly located in the marginal zone (MZ) area, surrounding the T-cell enriched lymphoid follicle (F). (E) FCM analysis of plasmacytoid DC specific markers expression on splenocytes harvested from Aire-Cre:Rosa26R-EYFP mice. Cells were gated on the YFP^{+}MHC II^{high} population. Representative FCM result of six independent experiments is shown. (F) RT-qPCR analysis of insulin expression in P1 + P2 and P3 populations of cells, in comparison to pancreatic islets. Cells were harvested from a pool of three spleens from three separate experiments. *Left panel*, realtime RT-PCR amplification plot shows cycle number versus normalized reporter (Rn) fluorescence. The original islet cDNA samples were diluted 10 and 1000 times before used as input template for the amplification of *Hprt* and *Insulin*, respectively. *Right Panel*, levels of *Insulin* mRNA expression relative to *Hprt*. As shown, the number of insulin transcripts in P3 is approximately 1–2% of that of *Hprt*, and about 0.001% of the pancreatic islets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pDC-specific marker mPDCA1 (Fig. 3D and Supplementary Fig. 2), suggesting that the Aire-expressing cells may represent a novel subpopulation of tolerogenic APCs.

Histologic examination revealed that EYFP$^+$ cells were primarily located in the marginal zone surrounding the lymphoid follicle (Fig. 3E), similar to tolerogenic DCs described previously [34]. RT-qPCR analysis of insulin gene expression in P1, P2 and P3 populations showed that insulin transcripts were essentially undetectable in either P1 or P2 populations, encompassing the majority of the conventional DCs and the CD11c^{int}EYFP$^-$ NKDCs, respectively [25]. In contrast, insulin transcripts were predominantly present in the P3 population, indicating that, unlike thymus and lymph nodes, insulin expression in the spleen is largely restricted to Aire$^+$ APCs of BM origin (Fig. 3F).

3.4. Deletion of ectopic insulin expression in CD11c$^+$ DC subsets is not sufficient to induce anti-islet autoimmunity

Only 85–90% of splenic CD11c$^+$ cells were positive for EYFP in Vav-Cre:Rosa26R-EYFP mice, in contrast to >95% cells of other hematopoietic lineages (Supplementary Fig. 1B). Thus, the absence of anti-insulin autoimmunity in Vav-ΔIns mice could be attributed to inefficient Cre-mediated deletion of the floxed *Ins2* gene in tolerogenic CD11c$^+$ DC subsets. To specifically delete *Ins2* in Aire$^+$CD11c^{int}B220$^+$ DCs in the spleen, we crossed CD11c-Cre transgenic mice to Ins1$^{del/del}$:Ins2$^{flox/flox}$ mice to generate the CD11c-ΔIns mice (genotype: CD11c-Cre: Ins1$^{del/del}$:Ins2$^{flox/flox}$). The CD11c-Cre transgene has been shown to be able to mediate efficient deletion of the floxed genes in all splenic DC subsets, including CD11c^{Int} pDCs, CD8$^-$ and CD8$^+$ DCs, as well as tissue-derived DCs in the lymph nodes, but not in NKDCs [39]. Similar to Vav-ΔIns mice, CD11c-ΔIns mice remain euglycemic throughout life, with no sign of compromised glucose metabolism or islet dysfunction (data not shown). Thus, under these conditions, deletion of insulin expression in CD11c$^+$ cells does not induce anti-islet autoimmunity.

3.5. B6^{g7}·CD11c-ΔIns mice display elevated levels of islet autoimmunity

Like Vav-ΔIns mice, CD11c-ΔIns mice are also in C57BL/6 background with intact central negative selection. Conceivably, insulin-reactive T-cells can be efficiently eliminated within the thymus. To examine the roles of insulin expression in Aire$^+$CD11c^{int} DCs in peripheral immune tolerance, we crossed the CD11c-ΔIns mice with B6.H-2^{g7} congenic mice, which carry the autoimmune diabetes-prone H-2^{g7} MHC allele of the NOD mice, to generate the B6^{g7}·CD11c-ΔIns mice. Normal levels of blood glucose were observed (Fig. 4A and data not shown). In addition, no difference of hematocrits was observed between B6^{g7}·CD11c-ΔIns mice and littermate controls, as well as age and gender matched Vav-ΔIns mice (Supplementary Table 1). When challenged with intraperitoneal glucose tolerance test (IPGTT), no difference of glucose metabolism was observed between the B6^{g7}·CD11c-ΔIns mice and the B6^{g7} littermate controls (Fig. 4B), suggesting the existence of sufficient islet function to maintain glucose homeostasis. Consistent with previous reports, a low degree of immune cell infiltration in the islets (insulitis) was observed in B6^{g7} controls (Fig. 4C, top panels). However, both the severity and the percentage of islets infiltrated by T cells were significantly increased in B6^{g7}·CD11c-ΔIns pancreata (Fig. 4C, lower panels). As illustrated in Fig. 4D, about 50% of islets were infiltrated with CD4$^+$ T cells, as compared with less than 20% in controls (53.6 ± 11.3% vs. 16.2 ± 4.0%, $p < 0.05$). Elevated levels of islet infiltration of CD8$^+$ T cells were also observed (46.0% ± 6.9% vs. 17.4 ± 4.1%, $p < 0.05$). These data suggest that insulin-expressing, Aire$^+$CD11c^{int} tolerogenic DCs may play a role in controlling the activation and expansion of insulin-reactive CD4$^+$ effector and CD8$^+$ cytotoxic T cells in the periphery. Of note, no change of T regulatory (T_{reg}) cells (both percentage and absolute number) was observed in both the pancreatic lymph nodes and spleens of the B6^{g7}·CD11c-ΔIns mice, in comparison to B6^{g7} controls (data not shown), suggesting that insulin expression in CD11c$^+$ cells mediates peripheral β-cell tolerance primarily through immune tolerogenic mechanisms other than increasing the total number of T_{reg} cells at inflammation sites.

4. Discussion

Thymic expression of organ-specific autoantigens, such as insulin, has been shown to be essential for negative selection of autoreactive T-cells and therefore for prevention of autoimmunity. Using the ID-TEC animal model, we have previously demonstrated the pivotal roles of insulin expression in medullary thymic epithelial cells (mTECs) in establishing central tolerance of pancreatic β-cells. However, one caveat in our previous study was that low levels of insulin transcripts, as well as *Aire*, were also found in a small population of APCs of hematopoietic origin [21,32,33,40]. Therefore, insulin deletion in Aire-expressing thymic DCs could also contribute to the faulty negative selection of insulin-reactive T cells in ID-TEC mice. Our present results on Vav-ΔIns and CD11c-ΔIns mice demonstrate that in the context of intact insulin expression in mTECs, its abrogation in BM-derived APCs is not sufficient to induce anti-insulin autoimmunity. Thus, at least in the case of insulin, thymic expression of pancreatic β cell-specific genes in mTECs plays a dominant role in establishing central self-tolerant of β-cells.

Nevertheless, transgenic overexpression of (pro)insulin in BM-derived APCs has been shown to be able to halt the progression of insulitis, and prevent the onset of clinical diabetes in NOD mice [41]. Similarly, transplantation of bone marrow progenitor cells transduced with (pro)insulin-expressing retroviruses into myeoablative pre-diabetic NOD recipients can effectively reverse the anti-islet autoimmunity, and protect them from developing diabetes [42]. These studies, in conjunction with the observation that insulin is expressed in secondary lymphoid organs of naïve animals, argue for a contributory role of BM-insulin expression in modulating peripheral immune tolerance of pancreatic β-cells. Taking advantage of the ability of Aire-Cre:Rosa26R-EYFP to mark Aire-expressing cells with detectable fluorescent signal, we were able to successfully identify the insulin-expressing cells in the spleen as a population of BM-derived Aire-expressing (EYFP$^+$) MHC II$^+$CD11c^{int}B220$^+$Gr1$^+$ DCs.

Tolerogenic DCs with similar surface markers (MHC II$^+$CD11c^{int}B220$^+$Gr1$^+$) as Aire-expressing splenocytes have been implicated previously in mediating β-cell tolerance. Kared et al. have shown that treating prediabetic NOD mice with granulocyte colony-stimulating factor (G-CSF) induced a marked increase of CD11c^{int}B220$^+$ DCs, resulting in prevention of spontaneous diabetes in NOD mice [43]. Steptoe et al. cultured NOD BM cells that expressed proinsulin controlled by a MHC class II promoter with GM-CSF and TGF-β and obtained a population of Gr-1$^+$ MHC II$^+$ tolerogenic DCs [44]. When these cells were adoptively transferred into 4-week old female NOD mice, significant suppression of diabetes development was observed. Although the specific role of CD11c^{int}B220$^+$ pDCs in T1D remains elusive [45,46], several reports showed a reduction of pDC numbers in blood of T1D patients, as compared with healthy controls [47,48]. In prediabetic NOD mice, pDCs exhibit impaired abilities to produce cytokines, such as interleukin 6 (IL-6) and granulocyte and macrophage-colony stimulating factor (GM-CSF) [49]. Thus, it is conceivable that diminished function of pDCs might contribute to the pathogenesis of

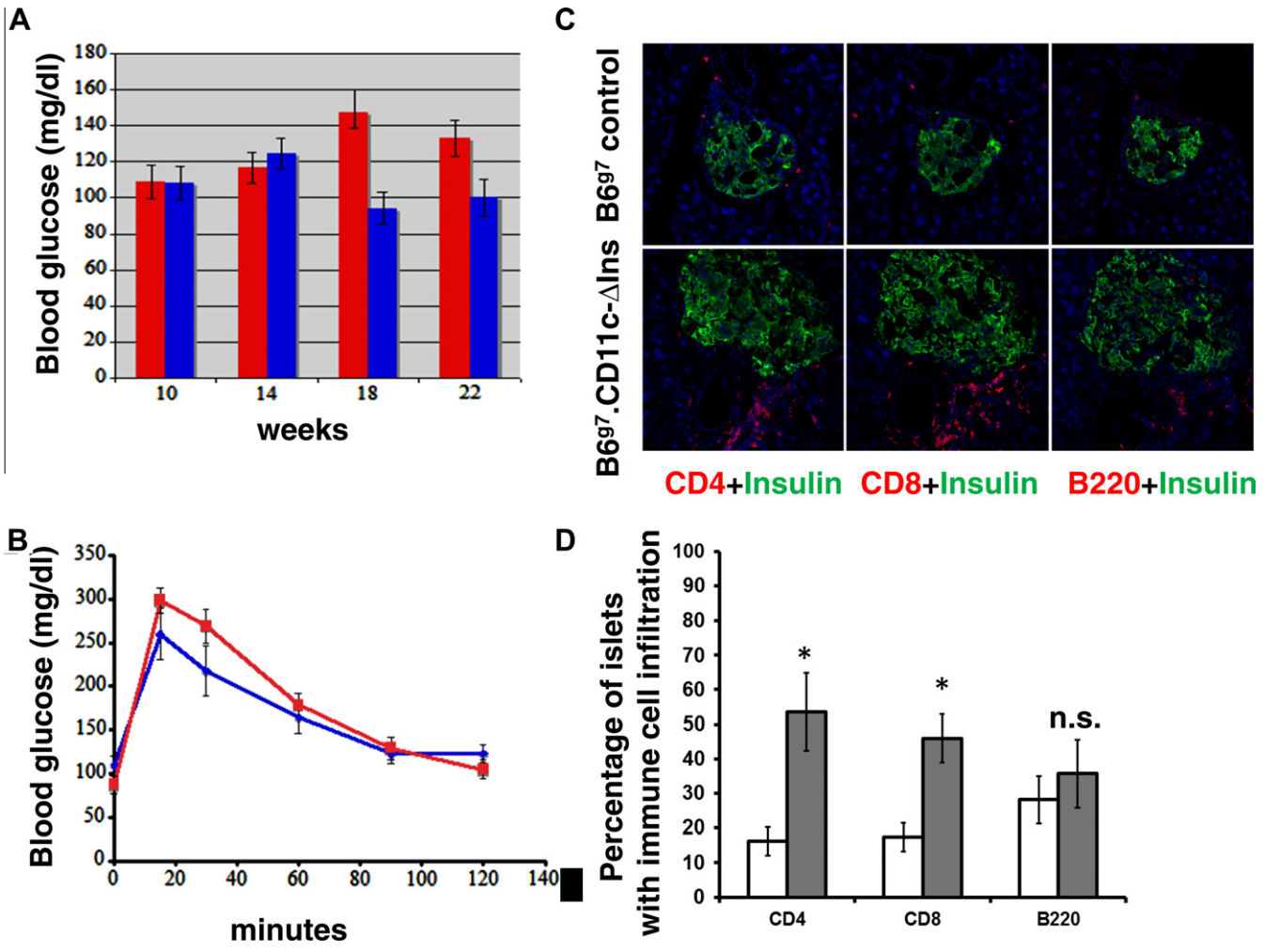

Fig. 4. $B6^{g7}$·CD11c-ΔIns mice displayed increased levels of islet autoimmunity. (A) Blood glucose levels were determined for $B6^{g7}$·CD11c-ΔIns mice (red bar, $n = 7$) and littermate controls (blue bar, $n = 7$). Similar results were obtained when the animals are 40 weeks old. (B) Intraperitoneal glucose tolerance test (IPGTT). $B6^{g7}$·CD11c-ΔIns mice (red line, $n = 7$, 40-week old) displayed normal glucose tolerant response when challenged with 2 g/kg bodyweight glucose, compared with $B6^{g7}$ littermate controls (blue line, $n = 7$). (C) Immunohistochemical analyses of pancreata harvested from 40-week old $B6^{g7}$·CD11c-ΔIns mice and non-Cre $B6^{g7}$ littermate controls. Shown are representative consecutive cryosections stained with anti-insulin antibody (green), in conjunction with anti-CD4 (left panels), anti-CD8 (middle panels) and anti-B220 (right panels) antibodies (red). Percentages of islets infiltrated with specific T lymphocytes ($CD4^+$ and $CD8^+$) and $B220^+$ cells are shown in (D). Five animals of each group were analyzed, and at least 10 islets were examined in each pancreas. $^*p < 0.05$, n.s., not significant.

autoimmune diabetes. Antibody-mediated depletion of pDCs in young BDC2.5/NOD transgenic mice drastically increased the severity of insulitis, consistent with a tolerogenic role of pDCs in modulating ongoing islet-autoimmunity [50]. Nevertheless, it remains to be determined whether the $Aire^+$ cells observed in Aire-Cre:Rosa26R-EYFP mice represent a specific population of tolerogenic DCs, or if acquiring Aire-expression is a common property of various subsets of APCs to exert their tolerogenic function.

Although we observed an increase of islet infiltration of both effector $CD4^+$ and cytotoxic $CD8^+$ T-cells, we did not find any decrease of T_{reg} cells in either pancreatic lymph nodes or spleens of the $B6^{g7}$·CD11c-ΔIns2 mice. Aire-expressing tolerogenic DCs might exert their inhibitory effect directly on the activation of insulin-specific autoreactive effector T cells. One of the mechanisms for tolerogenic DCs to modulate local T-cell response is to control the production of indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades the essential amino acid tryptophan [51–53]. Mellor and colleagues have shown that the suppressive action of MHC $II^+CD11c^+B220^+$ tolerogenic DC on T-cell response to allo-antigens depends on IDO production [54]. We have shown previously that adenovirus-mediated IDO over-expression can prolong the survival of transplanted islets in diabetic NOD mice by inhibiting the proliferation of islet-specific autoreactive T cells [55,56]. Thus, it is conceivable that engagement with insulin-reactive T-cells might stimulate insulin-expressing tolerogenic DCs to up-regulate immune suppressing modulators, such as IDO, to inhibit local auto-reactive T-cell expansion in the pancreas.

Alternatively, Aire-expressing tolerogenic DCs might be activated to produce tolerogenic cytokines, such as IL-10, and render insulin-reactive T cells anergic [57]. As an inflammatory disease of the pancreatic islet, T1D is characterized by the expansion of islet-reactive, IFN-γ-producing Th1 cells, driven by islet autoantigen-presenting APCs over-producing pro-inflammatory cytokines, such as IL-12. Paradoxically, neither IFN-γ nor IL-12 is indispensable for T1D development, indicating levels of redundancy of pro-inflammatory cytokines in mediating β-cell destruction (reviewed in [58]). Recently, IL-21 was identified as one of the critical cytokines for mediating β-cell autoimmunity in T1D, as IL-21R deficient NOD mice were almost completely protected from insulitis and diabetes [59]. Furthermore, it was shown that dysregulation of IL-21 signaling in APCs is of pivotal importance to the observed increase of islet-specific, pathogenic Th17 subsets of $CD4^+$ T cells in NOD mice [60]. Interestingly, it was shown recently that APC-production of pro-inflammatory cytokine IL-18 induces a

cytokine production switch (from IL-17 to IFN-γ) in autoreactive naïve T cells upon islet autoantigen encounter, consequently promoting their terminal differentiation into Th1 effector cells and early expansion [61]. In contrast to conventional DCs, engagement of islet-antigen presenting Aire-expressing DCs might inhibit these early activation and expansion events of autoreactive naïve T cells.

As a complex, chronic autoimmune disorder, the initiation and progression of T1D is the result of the breakdown of immune tolerance mechanisms at multiple immunomodulatory check-points, including but not limited to, faulty negative selection at the central level, defective tolerogenic DC function in the periphery, or insufficient T_{reg} cell suppression. Our data indicated that ectopic expression of (pro)insulin in immune organs plays efficient yet different immunomodulatory roles to maintain self-tolerance. (Pro)insulin expression in mTECs is essential to establish, at a central level, a tolerant T cell repertoire through depletion of insulin-specific autoreactive T cells, whereas insulin expression in $Aire^+$ tolerogenic DCs, as shown in the current study and from other groups, might exert maintenance roles of islet self-tolerance by suppressing the expansion of autoreactive T cells that have escaped the central negative selection. Thus, understanding the factors that govern the transcriptional regulation of insulin ectopic expression in specific antigen presenting stromal cells (mTECs or $Aire^+$ APCs) as well as their polymorphisms would help to explain the clinical diversity of T1D progression in affected individuals. While it is technically challenging to genetically engineer the thymus for rehabilitation purposes, gene therapies targeting specific subsets of tolerogenic DCs have shown promise for halting ongoing anti-islet autoimmune responses and prevention of diabetes in both NOD mice and humans [62,63]. Thus, strengthening tolerogenic mechanisms through genetic engineering of BM-derived $Aire^+$ insulin-expressing APCs seems to be a more practical therapeutic approach than intervention at the thymic level to regain islet-autoantigen-specific peripheral tolerance towards β-cells and halt disease progression.

Acknowledgments

We thank Dr. Jerry Adams for the Vav-promoter vector. We also thank Robert Lakomy, Alexis Styche, Joseph Flounlacker and Catarina Wong for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellimm.2011.12.010.

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