Prevention of diabetes by FTY720-mediated stabilization of peri-islet tertiary lymphoid organs

Running title: Diabetes prevention by TLO stabilization

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Submitted 30 July 2009 and accepted 1 March 2010.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

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Objective The nonobese diabetic (NOD) mouse is a well-established mouse model of spontaneous type 1 diabetes, which is characterized by an autoimmune destruction of the insulin-secreting pancreatic β-cells. In this study, we address the role of tertiary lymphoid organs (TLOs) that form in the pancreas of NOD mice during disease progression.

Methods We developed a model designed to “lock” lymphocytes in the pancreatic lymph node (PLN) and pancreas by the use of FTY720, which blocks the exit of lymphocytes from lymph nodes. A combination of flow cytometry, immunofluorescence, and analysis of clinical scores was used to study the effects of long-term FTY720 treatment on TLO development and development of diabetes.

Results Continuous treatment of NOD mice with FTY720 prevented diabetes development even at a time of significant insulitis. Treatment withdrawal led to accelerated disease independent of the PLN. Interestingly, naïve T cells trafficked to and proliferated in the TLOs. In addition, morphological changes were observed that occurred during the development of the disease. Remarkably, although the infiltrates are not organized into T/B cell compartments in 8-week old mice, by 20 weeks of age, and in age-matched mice undergoing FTY720 treatment, the infiltrates showed a high degree of organization. However, in naturally and FTY720-induced diabetic mice, T/B cell compartmentalization was lost.

Conclusion Our data show that TLOs are established during diabetes development and suggest that islet destruction is due to a loss of TLO integrity, which may be prevented by FTY720 treatment.
The nonobese diabetic (NOD) mouse is a well-established mouse model of spontaneous type 1 diabetes, which is characterized by an autoimmune attack against the insulin-secreting pancreatic β-cells in the islets (1). Multiple factors play a role in the development of diabetes in the NOD mouse model starting with the activation of T cells by antigen-presenting cells, leading to T cell differentiation and, ultimately, destruction of the target tissue. However, the location of these key events remains unclear. Lymphocytic infiltrates can be detected surrounding the islets in NOD mice as early as 3 to 4 weeks of age (2). The infiltrate continues to amass and by 15 to 18 weeks of age completely invades the islet and destroys it. Although virtually all NOD mice show some level of insulitis, only 60 to 80% of females and 20 to 30% of males, depending on the colony, develop fulminant disease (1). Therefore, the autoimmune destruction of pancreatic islets in NOD mice has been suggested to include two phases: insulitis, which involves infiltration of lymphocytes cells into the pancreas, and overt diabetes, which involves killing of the β-cells in the islets and resultant hyperglycemia (3). Interestingly, it has been shown that removal of the draining pancreatic lymph node at 3 weeks of age protects from disease development but removal at 10 weeks of age has no effect on disease development, suggesting that disease can become lymph node independent (4).

Organized lymphocytic infiltrates forming ectopically in non-lymphoid tissues have been termed tertiary lymphoid organs (TLOs) because of their morphological similarities to secondary lymphoid organs (5). These include the presence and compartmentalization of T and B cells controlled by chemokine gradients, high endothelial venules (HEVs), lymphatic vessels and antigen-presenting cells. TLOs have been described in a variety of autoimmune diseases including gastritis, thyroiditis, and systemic lupus erythematosus (5). However, a detailed analysis of TLOs in the pancreas of NOD mice has not been performed and could give insights into the development of the disease including whether antigen presentation occurs in the pancreas leading to epitope spreading and expansion of the immune response; whether continuous recruitment of lymphocytes to the site is necessary to cause disease and whether priming of T cells in these structures sufficiently activates them to become pathogenic. In this study, we used the S1P1 agonist FTY720, which blocks T cell egress from lymphoid tissues (6), to “lock” cells in the draining pancreatic lymph node and the pancreas to study the role of TLOs in the pathogenesis of diabetes in NOD mice.

RESULTS

Continuous FTY720 treatment prevents diabetes but leads to rapid disease development upon withdrawal: FTY720 blocks T cell egress from lymph nodes and has been shown to be effective clinically in the treatment of autoimmunity and transplant rejection (7). We set up a model using FTY720 that was designed to examine the effects of “locking” lymphocytes in the pancreatic lymph node (PLN) and pancreas. Treatment of female NOD mice with 20μg/day 3 times per week starting at 10 or 14 weeks of age, when insulitis had already begun or was established, completely protected mice from diabetes development (Figure 1A,B). Withdrawal of drug treatment after 10 or 7 weeks, respectively, led to rapid development of diabetes. Seventy to eighty-eight percent of mice were diabetic 18 days post-treatment withdrawal in multiple independent experiments (Figure 1A, B). This compares to 48% incidence among untreated
control mice. Similarly, continuous treatment until 25 weeks led to protection during treatment and acceleration of disease upon withdrawal (data now shown). In order to assess whether insulitis at the beginning of treatment was necessary for the rapid disease onset seen after withdrawal, we began treatment at 7 weeks of age, when insulitis is present but minimal (Figure 1C). Unlike the previous regimens, withdrawal 10 weeks later did not lead to rapid diabetes development. By 29 weeks of age, 12 weeks after FTY720 withdrawal, only 50% of FTY720 treated mice had become diabetic with similar kinetics as observed in control mice. As expected, continuous FTY720 treatment led to a marked decreased of circulating lymphocytes which returned to normal levels 7 days after treatment withdrawal (data not shown). These results suggest that the disease acceleration was not due to direct effects of the drug on the pancreas.

**Continuous FTY720 treatment does not clear pancreatic infiltrate:** A possible explanation for disease protection during FTY720 treatment is clearance of pancreatic infiltrate. Histological analysis and scoring of islet infiltrates of 20-week old mice that had been treated with FTY720 from 10-12 to 20-22 weeks of age and age-matched controls showed that treatment had limited effects on islet infiltration (Figure 1D). Forty percent of the islets had some degree of peri-islet infiltration similar to that observed in untreated 10-12 week old NOD mice suggesting that FTY720 treatment did not clear tissue infiltrate but rather blocked trafficking of new cells from the periphery. Interestingly, the overall fraction of pancreatic islets exhibiting grades 1, 2 or 3 insulitis remained consistent between 10-week old and 20-week old FTY720-treated mice suggesting that FTY720 blocked further islet invasion.

**FTY720 treatment leads to minor changes in infiltrating CD4+ T cells:** Next, we compared the infiltrate in 20-22 week old mice undergoing continuous FTY720 treatment from 10-12 weeks of age (as in Figure 1A) to aged-matched untreated controls. The total number of cells in the non-draining inguinal lymph node (ILN) and the spleen was 3-fold less in FTY720 treated mice (Table 1) likely due to sequestration of cells in the thymus (data not shown) and systemic lymphopenia, respectively. Interestingly, the total cell number in the PLN remained unchanged (Table 1); a result that could reflect continued expansion of cells specific for pancreatic antigens. Similar results were observed when examining the CD4+ and CD8+ T cells (Table 1). In the pancreas, the percentage of CD4+ T cells decreased 2-3 fold but there was no change in infiltrating CD8+ T cells (Table 1). These results suggest that disease protection during treatment, but not the disease acceleration seen after withdrawal, may be due to a decrease in infiltrating CD4+ T cells. Importantly, these data suggest that potentially pathogenic T cells were able to persist at the site of inflammation without continuous trafficking from peripheral lymphoid tissues.

Since FTY720 has been suggested to increase the proportion of Tregs (8; 9), we assessed whether a proportional increase in Tregs could account for the disease protection during treatment. Analysis of absolute numbers of Tregs showed that the total number of CD4+Foxp3+ cells decreased in the ILN and spleen but remained the same in the PLN (Table 1). The percentage of CD4+Foxp3+ cells in the pancreas remained the same; however, the absolute number of Tregs likely decreased in treated mice suggesting that changes in Tregs were unlikely to be responsible for disease prevention.

In addition to inhibiting the migration of lymphocytes, FTY720 has also been suggested to have effects on dendritic cells
(DCs) (10). To test this hypothesis, we treated 16-18 week old NOD mice with FTY720 for 10 days and analyzed the percentage of CD11c+ cells in the islets, PLN and ILN as well as their expression of CD86, CD40, CD80 and I-A^g7 (MHC II). The treatment did not affect the percentage of DCs or their maturation state in any tissue analyzed (Supplemental Figure 1 which can be found in an online appendix at http://diabetes.diabetesjournals.org), suggesting that disease protection observed during FTY720 treatment or disease acceleration observed after treatment withdrawal was not due to changes in DCs.

**Analysis of tertiary lymphoid organs in the pancreas:** Since the total cell number in the PLN was unaffected by FTY720 treatment and a significant number of cells remained in the pancreas, it was unlikely that disease protection during FTY720 treatment could be attributed solely to a reduction in infiltrating cells. Therefore, we analyzed the effect of long-term FTY720 treatment on the structure and characteristics of TLOs. Because FTY720 blocks lymphocyte egress from the lymph nodes, we hypothesized that disease was prevented by inhibiting lymphocyte egress from the TLOs in the pancreas to the site of tissue destruction - the β-cells. First, we characterized the TLOs in pre-diabetic 8-week old, non-diabetic 20-week old and naturally diabetic mice by immunofluorescent staining of individual markers on consecutive pancreas sections using markers described in the Materials and Methods.

ERTR7 reactivity and CD31+ vessels were observed surrounding all islets and throughout the acinar tissue at all the time points analyzed (Figure 2A and data not shown). In addition, ERTR7 reactivity was found within the infiltrate suggesting that these structures have a supporting stromal network, as is seen in T cell zones in the spleen and lymph nodes (11). However, although the results trend towards an increase in the association of the presence of MAdCAM^+ or Lyve^+ vessels and disease progression, these differences were not statistically significant (Supplemental Figure 2B). In addition, most infiltrates in both pre-diabetic and naturally diabetic mice contained PNAd^+ HEVs (Figure 2D-G) suggesting that, like secondary lymphoid organs, these structures are capable of recruiting naïve T cells. Analysis of at least three consecutive sections showed that infiltrates of pre-diabetic 20-week old mice were usually, but not always, associated with insulin-producing islets (Figure 2A and 2E). Although T and B cells were present in the pancreas of 8-week old mice, only 15% (3/20) of the infiltrates showed clear compartmentalization whereas 89% (24/27) infiltrates in 20-week old mice showed distinct T and B cell zones. In the majority of these, the T cell zone was closer to the islet while the B cell zone was on the edge of the infiltrate, reminiscent of lymph nodes (Figure 2A). Only 15% of infiltrates (3/19) in naturally diabetic mice showed T/B cell compartmentalization, which correlated with an absence of insulin-positive islets that we attribute to destroyed β-cells (Figure 2B and 2G). However, β-cells may have been present but not producing insulin due to the inflammation (12). True TLOs, defined as infiltrates with T/B compartmentalization and association with at least two of the vascular markers analyzed, were present in 15% (3/20) of infiltrates in 8-week old mice increasing to 81% (22/27) of infiltrates in 20-week old mice but decreasing to only 26% (5/19) of infiltrates in naturally diabetic mice (Figure 2D-F). Therefore, TLOs peaked as a proportion of the infiltrate at the “peak” of the inflammatory response before diabetes onset.

We analyzed NOD-Rag KO mice to assess whether the appearance of the TLO markers was due to inflammation or a normal characteristic of the NOD pancreas (Figure 2C). As in NOD mice, there was ERTR7
staining and CD31+ vessels around islets and throughout the acinar tissue. MAAdCAM+ and Lyve-1+ vessels were associated with 30% and 59% of islets, respectively, which is significantly lower than in mice with an intact immune system (Supplemental Figure 2B). There was no PNAd staining suggesting an adaptive immune response was necessary for the development of HEVs in the pancreas (data not shown).

Analysis of FTY720 treated mice after 8-10 weeks of continuous treatment (20-weeks old) showed that the majority of the infiltration was associated with insulin-producing islets (Figure 2G). Moreover, the infiltrates in FTY720 treated mice showed a similar degree of organization, based on T/B compartmentalization (19/21), as age-matched untreated controls (Figure 2G). Similar to pre-diabetic and naturally diabetic mice, the TLOs in these mice had PNAd+ HEVs as well as Lyve-1+ and MAAdCAM+ vessels (Supplemental Figure 2A). Analysis of mice on day 12 after FTY720 release, when 30-60% mice have become diabetic, showed that the infiltrates lose T/B compartmentalization and are similar to those of naturally diabetic mice; only 31% (5/16) of infiltrates were true TLOs based on our criteria (Figure 2H).

In an attempt to define TLOs in humans with type 1 diabetes, we analyzed pancreatic sections from four such patients obtained through JDRF’s Network for Pancreatic Organ donors with Diabetes (nPOD; www.jdrfnpod.org). Although CD4+ T cells and CD19+ B cells were found surrounding insulin+ islets (Supplementary Figure 3), the cells were scattered and did not form large clusters as was observed in prediabetic and early-onset diabetic NOD mice. However, at present, we cannot distinguish whether the absence of TLOs in the human samples is due to temporal differences in sampling (i.e., the human tissues represented those obtained from individuals with longer disease duration than those we analyzed in NOD mice) or actual differences between the disease as it is expressed in the two species.

Accelerated disease upon FTY720 withdrawal is independent of the draining pancreatic lymph node: A possible explanation for the accelerated disease following cessation of FTY720 is that the relevant antigen-specific cells continue to expand to sufficient numbers and activation status to cause rapid disease upon treatment withdrawal and restoration of trafficking. This would suggest that during long-term FTY720 treatment there are reservoirs of islet-reactive cells that accumulate either within or outside of the pancreas. To determine in which lymphoid tissues islet antigens are presented that can lead to antigen-specific T cell expansion, we transferred 1 x 10^6 CFSE labeled naïve CD4+CD25-CD62L+ islet antigen-specific BDC2.5 TCR-Tg cells to 8-week old NOD mice and treated with FTY720 daily to prevent recirculation. After 5 days, cells were harvested and proliferation evaluated by CFSE dilution (Figure 3). In untreated mice, 37% and 50% of transferred cells had entered the cell cycle in the PLN and the pancreas, respectively. The proliferation was antigen dependent as only 8% and 14% of the TCR-Tg T cells went into cycle in the ILN and spleen, respectively. In FTY720 treated mice, a higher percentage of cells that entered the cell cycle was observed in the PLN (54%) and pancreas (75%). These results suggested that naïve cells were able to home directly to and proliferate in the pancreas and that FTY720 treatment did not prevent antigen presentation or antigen driven proliferation. Similar to untreated mice, 6% and 2% of the cells in the ILN and spleen, respectively, proliferated in FTY720 treated mice suggesting that the PLN is the only secondary lymphoid tissue where islet antigens are presented and that the
proliferating cells seen in the spleens of untreated mice are a result of recirculation.

We reasoned that the cells causing rapid onset of diabetes upon FTY720 withdrawal originated from either the PLN or the pancreas. Thus, we tested the effect of surgical removal of the PLN in mice undergoing FTY720 treatment. FTY720 treatment was begun at 12 weeks of age, the PLN was surgically removed (PLNx) 9 weeks later, and treatment was withdrawn one week after the surgery. Forty percent of PLNx mice developed diabetes within 15 days of treatment withdrawal compared to 80% of sham controls (Figure 4A). As our previous experiments had shown, the age at the beginning of treatment influences whether there is accelerated disease upon treatment withdrawal suggesting that although the PLN may be important early in disease progression, the requirement of this lymph node diminishes as disease progresses. We hypothesized that if we began treatment late in life, a greater percentage of mice would develop disease independently of the PLN due to the increased level of infiltration at the time of treatment. Therefore for the second series of experiments, we began treatment at 16 weeks of age, removed the PLN at 23 weeks of age and withdrew FTY720 one week later. Under these conditions, 50% of sham control mice and 65% of PLNx mice became diabetic within 15 days of FTY720 withdrawal (Figure 4B). These results suggest that the diabetes acceleration observed after FTY720 can be attributed to antigen-reactive T cells present in both pools. However, the PLN is not required for the accelerated disease in mice with the most progressed disease. In this setting, the cells present in the TLOs of the pancreas at the beginning of the treatment are sufficient to cause diabetes upon FTY720 withdrawal.

**DISCUSSION**

In this study, we used FTY720 treatment of NOD mice to prevent trafficking of lymphocytes into the pancreas and study the role of tertiary lymphoid organs (TLOs) in diabetes development. Our results show that continuous FTY720 treatment prevents diabetes development and, when withdrawn, leads to an accelerated disease that is independent of the PLN if treatment is begun when insulitis is established. Analysis of NOD mice at various stages of disease and during FTY720 treatment showed that lymphocyte infiltration into the pancreas leads to the formation of organized TLOs characterized by T/B compartmentalization and specialized vasculature, including lymphatic vessels, HEVs and MAdCAM expressing vessels. Furthermore, our data showed that these structures do not exhibit T/B compartmentalization in naturally diabetic mice even though the specialized vasculature remains unchanged. We propose a model where cells that infiltrate the pancreas organize into TLOs that, like secondary lymphoid organs, have T/B compartmentalization and specialized vasculature. Once these structures are established the immune response is amplified and eventually is sufficient to destroy the neighboring islet. In doing so, T/B compartmentalization is lost and TLO integrity is lost.

Although there is agreement that the main mode of action of FTY720 is by blocking lymphocyte egress from the thymus and lymph nodes, some controversy remains concerning other effects on T cells. Indeed, protection from disease development even in the presence of significant infiltration in the pancreas suggested the possibility of direct effects of FTY720 on T cells. Previous studies have suggested that FTY720 induces Tregs or enhances their function (8,9). In our study, increased Tregs were only observed in the ILN, not in the PLN or in the pancreas, the locations where an increase in Tregs
would be likely to have an effect on disease outcome. Interestingly, it is unclear whether this increase was due to preferential homing, proliferation or conversion from effectors to Tregs. FTY720 has also been suggested to induce apoptosis of T cells (7). Similar to our results, Maki et al reported that continuous treatment of NOD mice with FTY720 prevented diabetes development during treatment (13). They observed diabetes protection even after withdrawal in mice that started treatment at 4 weeks of age which led to the suggestion that therapy eliminated autoreactive effector T cells by FTY720-mediated apoptosis (13). Rather than previous interpretations, we propose that the lack of accelerated disease following treatment of young prediabetic NOD mice is a result of a low number of antigen-specific T cells in TLOs at the time of treatment initiation, which was not sufficient to develop a pathogenic T cell population capable of rapid disease induction once drug treatment was discontinued. This interpretation fits with the analysis of 8-week old mice, where TLOs were not yet organized into distinct T and B cells zones and therefore not fully established by this point. Moreover, in a model of allograft rejection, Habicht et al showed that FTY720 treatment had no effect on alloreactive T cell apoptosis, T cell activation, proliferation, cytokine production (14). Our data are consistent with the documented mechanism of action of FTY720, namely, that FTY720 controls exit from primary and secondary lymphoid organs. Additionally, our data suggest that FTY720 also controls exit out of TLOs and entry into the neighboring pancreatic tissue thereby stabilizing these structures and preventing islet destruction and diabetes development.

Tertiary lymphoid organs have been previously described in the pancreas of NOD mice; however, a characterization at different stages of disease including association with specialized vessels had not been described. Our data show that Lyve1+ and MAdCAM+ vessels are present even in non-inflammatory conditions, such as the pancreas of NOD-RAG KO mice. Inflammation in NOD mice results in a higher percentage of islets associated with specialized vasculature. Whether this increase reflects neovascularization or expression of these markers on vessels that were previously present is not known.

The role of TLOs in the amplification of the immune response is unclear (15). Our results expand on the role of TLOs in T cell responses. The adoptive transfer of naïve BDC2.5 Tg T cells during FTY720 treatment shows that naïve cells can in fact be recruited directly to the pancreas rather than having to be primed first in the PLN. In addition, proliferation of these cells in the pancreas suggests that there is local antigen presentation (16). However, it is not known how the antigen reaches the TLOs. It is possible that islet-resident dendritic cells traffic to the TLO bringing antigen with them, antigen is picked up by TLO resident antigen presenting cells, such as macrophages and DCs, at the TLO/islet boundary or antigen travels through the lymph from the islet to the TLO.

The requirement of the lymphotoxin (LT) axis on TLO development has been explored because of its importance in lymph node development. Disruption of TLOs by over expression of LIGHT, a LTβR ligand, in the pancreas of NOD mice (NOD-RIP-LIGHT) results in accelerated diabetes compared to non-transgenic controls (17). This suggests that during the natural course of disease and TLO break down additional signals are present that allow pathogenic cells to destroy the islets rather than instructing them to leave the pancreas. Therefore, preventing islet destruction by altering TLO maintenance will require a deeper understanding of the signals that control islet destruction. In addition, targeting of TLOs
may be difficult since the same signals that induce, and presumably maintain, TLOs are also necessary for lymph node integrity (15).

Analysis of TLOs in type 1 diabetes patients has been challenging. In accordance with our findings, Willcox et al analyzed pancreas samples from patients who died within 18 months of diagnosis (19) and found scattered infiltrating cells surrounding glucagon or insulin positive islets. We hypothesize that the lack of true TLOs in the nPOD samples is due to timing: analyzing long-term clinically diabetic patients likely misses the peak of the immune response in the pancreas. In fact, the NOD mouse data supports this interpretation since we found 81% of infiltrates were localized to TLOs in 20-week old prediabetic mice but only 26% of infiltrates were localized to TLOs in diabetic mice. This highlights the need for screening and analysis of pre-diabetic, autoantibody-positive individuals in order to elucidate the role of TLOs in type 1 diabetes.

Promising clinical trials using FTY720 as a treatment for multiple sclerosis are underway (18) suggesting its possible use in treating other autoimmune diseases such as type 1 diabetes. However, our data suggest that autoreactive cells continue to expand during treatment and become highly pathogenic, such that if treatment were advertently or inadvertently discontinued the stealth T cells rapidly destroy the target tissue. This has not been documented thus far in the clinical trials; whether the rebound effect is restricted to diabetes or this mouse model, or whether it requires longer treatment remains to be seen.

**MATERIALS AND METHODS**

**Mice:** NOD, NOD.BDC25 (20) and NOD.Rag2 KO mice were bred at the University of California, San Francisco. NOD mice were also purchased from Taconic. All mice were housed under specific pathogen-free conditions at the University of California, San Francisco Animal Barrier Facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. For diabetes incidence, urine glucose was followed twice weekly.

**Human Pancreas Samples:** Samples from human diabetic patients were obtained through JDRF’s nPOD project. The age of the patients ranged from 12-22 years old and had been diagnosed with type 1 diabetes 1-8 years prior to death. Details regarding their collection and methods of preparation can be found at www.jdrfnpod.org.

**FTY720 administration:** FTY720 was obtained from Norvatis Pharmaceuticals and was dissolved in normal saline. A dose of 20µg/day was administered by i.p. injection three times per week.

**Pancreatic lymph node ectomy:** Pancreatic lymph nodes were removed as previously described (4).

**Reagents:** For flow cytometry, labeled antibodies specific for CD4 clone RM4-5, CD8 clone 53-6.7, CD25 clone PC61, FoxP3 clone FJK-16s, CD45 clone I3/2.3, CD11c clone N418, CD86 cone GL1, CD40 clone 3/23, CD80 clone 16-10A1, I-A\(^{g7}\) clone 10.2.16 were used. Cells were fixed and permeabilized using Foxp3 Staining Kit per manufacturer’s protocol. For immunofluorescence, primary antibodies specific mCD4 clone L3T4, hCD4 clone RPA-T4, mCD45R/B220-biotin clone RA3.6B2, hCD19 clone H1B19, mCD31 clone 390, mMadCAM clone MECA367, mLyve-1 clone 223322, and ERTR7 were used. MECA79 antibody was obtained from Dr. Steve Rosen (University of California, San Francisco).

**Immunofluorescence:** Pancreata were isolated and frozen in optimum cutting temperature embedding compound. Consecutive sections (7 \(\mu\)m) were cut with a Leica CM 3050S cryomicrotome and fixed for 15 minutes in acetone at -20°C. Sections
were blocked with 5% BSA and 3% normal serum corresponding to the secondary antibody and/or Avidin/Biotin Blocking Kit per manufacturer’s protocol. Slides were incubated with primary and secondary antibodies for 1-2 hours at room temperature and counterstained with Gill’s Hematoxylin. Digital images were captured with an Optiphot microscope equipped with an Axiocam digital camera and analyzed using Adobe Illustrator CS2.

Isolation of lymphocytes from pancreas: Lymphocytes were isolated from the pancreas as previously described (21)

Isolation of dendritic cells: Dendritic cells were isolated from lymph nodes and islets as previously described (22)

Adoptive transfer and CFSE dilution analysis: CD4+CD25−CD62L+ cells were sorted from NOD.BDC2.5tg*Thy1.1 mice. Cells were then labeled with CFSE and 1x10⁶ transferred to NOD.Thy1.2 mice. Immediately after transfer and daily until time of harvest, mice were injected with FTY720. On day 5 single cell suspensions from pancreas, spleen, PLN and ILN were stained for flow cytometric analysis. To calculate the percent of T cells that entered cell cycle, the original number of cells that entered cell cycle was determined by dividing the number of events at each division (i.e. CFSE peak) by the number of divisions that occurred. This number was divided by the total number of cells (original number of cells that entered cell cycle + number of cells that never entered cell cycle).

Statistical analysis: The statistical significance between Kaplan-Meier survival curves in Figure 1 and 4 was determined by a Logrank test using Prism software. The statistical significance of differences between groups in Table 1 and Figure 3 was determined by the two-tailed Student's *t* test using Excel. The statistical significance of differences between groups in Supplemental Figure 2 was determined by the Mann-Whitney test using Prism software.

ACKNOWLEDGEMENTS

The authors would like to thank Navdeep Grewal, Shuwei Jiang, Mark Singer, Patrick Rowe and Josh Beilke for technical assistance, Nicolas Martinier and Dorothy Fuentes for animal husbandry, Dr. Steve Rosen for the MECA79 antibody and Novartis Pharmaceuticals Corporation for FTY720. We thank Drs. Abul Abbas, Mark Anderson, Steve Rosen, Hans Dooms and members of the Bluestone laboratory for helpful discussions and reading the manuscript. This work was supported by National Institutes of Health Grants R37 AI46643 and P30 DK63720. nPOD tissues were obtained with support from the Juvenile Diabetes Research Foundation. CP was supported by NIGMS grant # 1 R25 GM56847.
REFERENCES


Table 1. Analysis of T cells during FTY720 treatment.

<table>
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<th>Pancreas</th>
<th>PLN</th>
<th>ILN</th>
<th>Spleen</th>
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<tr>
<td>Total cell number (x 10^6)</td>
<td>Control</td>
<td>n/d</td>
<td>2.73 ± 1.40</td>
<td>9.97 ± 3.50</td>
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<td></td>
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<td>n/d</td>
<td>3.16 ± 0.80</td>
<td>3.65 ± 1.03</td>
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<td>Percent CD4^+ of CD45^+ cells</td>
<td>Control</td>
<td>26.2 ± 9.6</td>
<td>48.5 ± 1.5</td>
<td>51.4 ± 7.5</td>
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<td></td>
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<td>32.4 ± 1.8</td>
<td>27.8 ± 3.3</td>
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<tr>
<td>Total CD4^+ cell number (x 10^6)</td>
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<td>n/d</td>
<td>1.32 ± 0.67</td>
<td>4.99 ± 1.47</td>
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<td>1.00 ± 0.25</td>
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<td>Percent Foxp3^+ of CD4^+ cells</td>
<td>Control</td>
<td>17.3 ± 6.8</td>
<td>21.6 ± 5.7</td>
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<td></td>
<td>FTY720 treated</td>
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<td>Total CD4^+Foxp3^+ cell number (x 10^6)</td>
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<td>n/d</td>
<td>0.28 ± 0.15</td>
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<td>Percent CD8^+ of CD45^+ cells</td>
<td>Control</td>
<td>7.2 ± 5.3</td>
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<td>Total CD8^+ cell number (x 10^6)</td>
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<td></td>
<td>FTY720 treated</td>
<td>n/d</td>
<td>1.01 ± 0.28</td>
<td>1.13 ± 0.26</td>
</tr>
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</table>

NOD female mice were treated for 10 weeks with FTY720 beginning at 10-12 weeks of age. Mice were analyzed on the last day of treatment. Total cell number and percentages of CD4^+ and CD8^+ lymphocytes amongst CD45^+ cells and CD4^+Foxp3^+ cells amongst CD4^+ cells was determined by flow cytometry. Mean values of 3 independent treatment experiments are shown. Control n=8. FTY720 treated n=11. n/d: not determined. PLN: draining pancreatic lymph node. ILN: non-draining inguinal lymph node. *p<0.01 between control and FTY720 treated in the same tissue.
Figure 1. FTY720 treatment of NOD mice. (A) Female NOD mice treated three times per week with 20µg/day FTY720 from 12 to 22 weeks of age (shaded). Control n=25, FTY720 treated n=10. p=0.0091 after FTY720 withdrawal. (B) Female NOD mice treated as in (A) starting at 14 weeks of age for 7 weeks. Control n=20, FTY720 treated n=8. p=0.0004 after FTY720 withdrawal. (C) Female NOD mice treated as in (A) starting at 7 weeks of age for 9 weeks (shaded). Control n=10, FTY720 treated n=15 pooled from two independent experiments. p=0.7723 after FTY720 withdrawal. (D) Histological analysis of pancreas sections at the end of FTY720 treatment in panel A. 10 weeks untreated n=6 mice, 1001 individual islets; 20 weeks untreated n=6 mice, 576 individual islets; 20 weeks FTY720 treated n=7 mice from two independent treatments, 816 individual islets. Grade 0=no infiltrate. Grade 1=<25% of islet infiltrated. Grade 2=25-75% of islet infiltrated. Grade 3=>75% of islet infiltrated.

Figure 2 Analysis of TLOs in the pancreas of NOD mice. Pancreas sections from (A) 20 week old, (B) naturally diabetic and (C) Rag2-KO NOD mice were co-stained with anti-insulin and anti-CD31, anti-ERTR7 and anti-insulin, anti-Lyve-1 and anti-insulin, anti-MAdCAM and anti-insulin, anti-CD4 and anti-B220 or anti-Lyve1 and MECA-79 antibodies. Representative immunofluorescence images of consecutive sections (except for C) stained with the indicated antibodies are shown on the top and are superimposed on hematoxilin counterstain bright field images on the bottom (green fluorophore appears as pink, red fluorophore appears as blue). (D-H) Infiltrates were scored based on their association with insulin producing islets (Column 1), whether there was T/B cell zone compartmentalization (Column 2), and association with Lyve-1+ (Column 3), MECA79/PNAd+ (Column 4) or MAdCAM+ (Column 5) vessels. Each row indicates a separate infiltrate/islet analyzed. Each block indicates a separate mouse. Bar represents 100µm. * indicates infiltrates considered to be true TLOs based on T/B compartmentalization and association with at least two of the vascular markers analyzed.

Figure 3. Proliferation of BDC2.5tg T cells in NOD mice. 1x10⁶ CD25−CD62L+ naïve BDC2.5 TCR transgenic cells were CFSE labeled and transferred to 8-10-week old NOD females. After transfer mice were treated with 20µg FTY720 daily. On Day 5 BDC2.5 proliferation was analyzed by CFSE dilution in the pancreas, draining PLN, non-draining ILN and spleen. (A) Representative histograms of control (top) and FTY720 treated (bottom) mice. (B) Percentage of cells that entered the cell cycle was calculated as described in the materials and methods section. Data pooled from two independent experiments. * p<0.01

Figure 4. Role of draining pancreatic lymph node on FTY720-induced diabetes. (A) NOD female mice were treated for 9-10 weeks (shaded) with FTY720 beginning at weeks 11-13. During the last week of treatment the pancreatic lymph nodes were surgically removed. Disease incidence was monitored after FTY720 withdrawal. Data are pooled from two independent experiments. Sham n=8, PLNx n=9. p=0.0817 (B) NOD female mice were treated for 8 weeks (shaded) with FTY720 beginning at week 16. During the last week of treatment the pancreatic lymph nodes were surgically removed. Disease incidence was monitored after FTY720 withdrawal. Sham n=6, PLNx n=8. p=0.8456.
Figure 1

A

B

C

D

Diabetes prevention by TLO stabilization

Figure 1

A

B

C

D
Figure 2

Diabetes prevention by TLO stabilization
Figure 3

A  

Pancreas  PLN  ILN  Spleen

Control  FTY720 treated

B  

Pancreas  PLN  ILN  Spleen

Cells that entered cell cycle

Control  FTY720 treated

Control  FTY720 treated

Control  FTY720 treated

Control  FTY720 treated
Figure 4

A

B