Regenerative Medicine Minnesota
Progress Report
Due: 8/30/2018

Grant Title: **Identifying and targeting antigen specific T cells in diabetes to preserve beta cells**

Grant Number: RMM 11215 TR002

Principal Investigator: Brian Fife, PhD

Project Timeline: 8/1/2016-7/31/2018

Progress to Date:

We have made significant progress in the grant objectives the past two years. Our efforts have resulted in two peer reviewed publications. In the first study, we used peptide:HLAII tetramer staining to compare the frequency and phenotype of InsB-specific $CD4^+$T cells directly ex vivo within peripheral blood from HLA-$DQ8^+$patients with T1D and HLA-matched control subjects without diabetes. We found that 54% (20 of 37) of patients with T1D had detectable insulin $tetramer^+$ cells compared with only 15% (4 of 26) of control subjects without diabetes (Fig. 1). Within the patients with T1D, 64% of insulin $tetramer^+$cells were antigen experienced ($CD45RO^+$). In fact, patients with the most $tetramer^+$ effector memory cells ($CD45RO^+$ $CCR7^-$) had significantly higher insulin antibody titers and the shortest T1D duration (Fig. 2). Importantly, $tetramer^+$ cells were enumerated from several patients with new-onset T1D where insulin administration was shorter than 15 days, providing evidence that these cells are self-reactive (not shown). In one subject without diabetes, a genetically at-risk first-degree relative of a patient with T1D, we found effector memory $tetramer^+$ cells in the absence of IAAs. Taken together, these data suggest that InsB-specific $CD4^+$ T cells become activated in response to endogenous antigen and may be contributing to antibody production. Determining their frequency and phenotype may be useful for assessing disease activity after diagnosis and potentially during the preclinical period of T1D development.

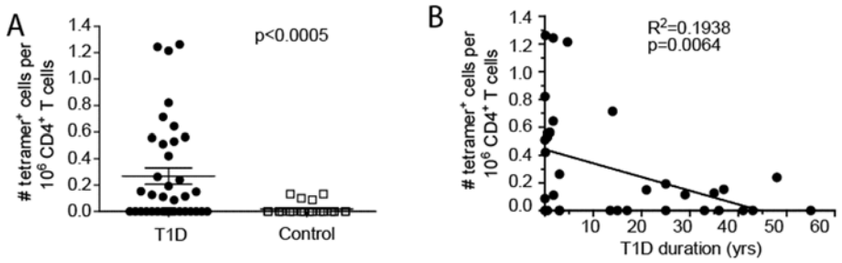

Figure. 1. Enumeration of islet specific $tetramer^+$ cells from T1D patients and non-diabetic individuals. (*A*) Frequency of islet specific-$tetramer^+$ cells per million $CD4^+$ T cells from all T1D patients (n=37) and healthy non diabetic controls (n=26). (*B*) Correlation of the frequency of $tetramer^+$ cells with disease duration for all T1D patients. (Data reproduced directly from Spanier et al. Diabetes. 2017 Dec;66(12):3051-3060.)

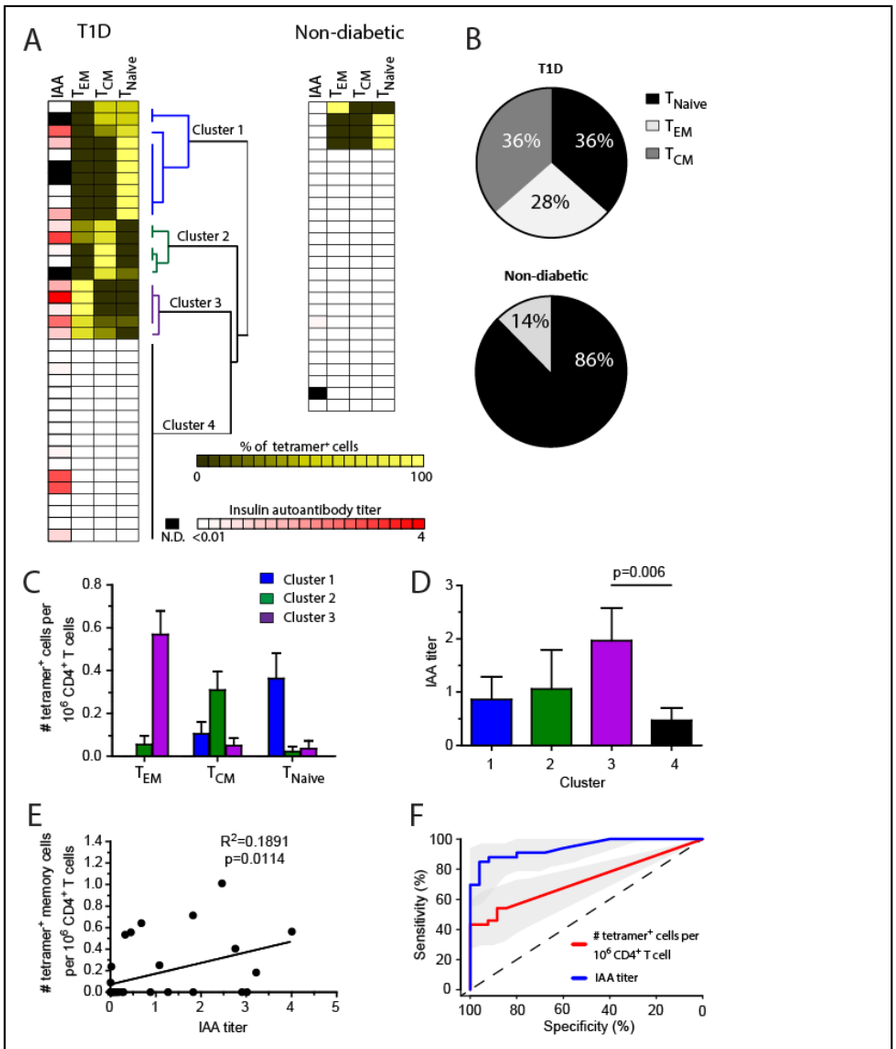

Figure. 2. Phenotype and correlation analysis of islet-specific-tetramer$^+$ cells with insulin autoantibody. (*A*) Insulin autoantibody (IAA) titer along with unbiased hierarchical clustering analysis based on tetramer+ cell frequency and phenotype. Each row represents an individual patient (n=37) or nondiabetic control sample (n=26), biological replicates, (N.D. = not determined, black box). (*B*) Combined phenotype of all tetramer$^+$ cells from all patients shown in (*A*). (*C*) Frequency of insulin-tetramer$^+$ cells within T_{EM}, T_{CM}, and T_{Naive} phenotypes from patients within each cluster as determined in (*A*). (*D*) Plasma IAA titer among patients within each cluster as defined in (*A*). (*E*) Correlation of the frequency of tetramer$^+$ memory (T_{EM} and T_{CM}) cells identified in each patient with their IAA titer. (F) Receiver operating characteristic (ROC) analysis using R (v.3.2.4) to measure the sensitivity, specificity, and overall performance of tetramer+ cell frequency or IAA to identify individuals having T1D.Bars represent the mean ± SEM and Student's T test (*D*) or linear regression (*E*) was used to determine significance. (Data reproduced directly from Spanier et al. Diabetes. 2017 Dec;66(12):3051-3060.)

In the second study, we measured islet β cell PD-L1 expression and regulation during diabetes pathogenesis. The goals of this study were to improve upon previous strategies for flow cytometric analysis of individual, insulin-positive, live β cells, and determine the specific regulators, location, and timing of PD-L1 expression in both mouse and human β cells. We utilized multicolor flow cytometry and epifluorescent microscopy to measure PD-L1 expression on islet β cells during spontaneous diabetes in NOD mice, and found that PD-L1 expression increased as mice approach diabetes onset, and was associated with islet infiltration (Fig. 3 and Fig. 4). We also investigated the effect of cytokines on PD-L1 expression. The CD274 promoter contains two interferon regulatory factor-1 (IRF-1) binding sites, and previous work has shown that type 1 and type 2 interferons (IFN) induce PD-L1 expression on T cells, B cells, endothelial cells, epithelial cells, and tumor cells. We found that IFN-γ and to a lesser extent, IFN-α, promoted increased frequency of PD-L1+ β cells, and increased expression on a per cell basis (Fig. 5). Similar to our findings in mice, within human pancreas we found that increased PD-L1 expression correlated with increased inflammatory T cell infiltration in pancreatic lesions (Fig. 6). Interestingly, we observed a minor increase in PD-L1 staining in autoantibody positive patients in the absence of overt autoimmune diabetes and found that Th1-associated cytokine IFN-γ modulated PD-L1 expression in vitro on isolated human islets (data not shown). Taken together, this work illustrates that both mouse and human islet β cells express PD-L1 in response

to the same inflammatory cues, which may help delay islet destruction, but is ultimately insufficient to prevent β cell death.

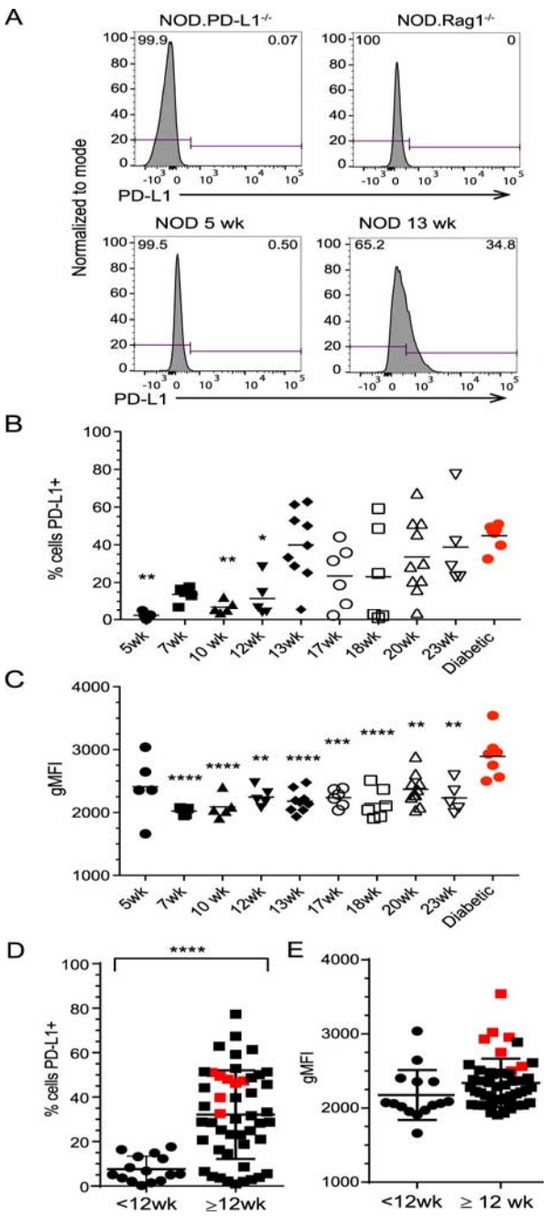

Figure 3. PD-L1 expression increases on β cells as NOD mice age and insulitis develops. (**A**) Shown are representative flow cytometry plots for control NOD.PD-L1$^{-/-}$ mice, lymphocyte-deficient NOD.Rag1$^{-/-}$ mice, and 5 or 13 week old NOD mice. NOD.PD-L1$^{-/-}$ mouse islets were used to set negative and positive gates for PD-L1 staining. (**B**) Shown is the frequency PD-L1^{+} insulin-positive β cells from each mouse as a percentage of all live insulin positive β cells in 5–23 week old mice as indicated or from diabetic NOD mice. Each symbol represents an individual mouse (n = 68). The mean is shown with a black line. (**C**) The PD-L1 geometric mean fluorescence intensity (gMFI) from each mouse in (**B**), (n = 68). The mean is shown with a black line. (**D**) The percentage of PD-L1^{+} insulin-positive β cells from younger (<12 wks) and older (≥12 wks) NOD mice from (**B**). (**E**) PD-L1 gMFI of PD-L1^{+} insulin-positive β cells from younger (<12 wks) and older (≥12 wks) NOD mice from (**C**). The lines in (**D**,**E**) represent the mean ± standard deviation. Significance was determined as compared to diabetic mice: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using One-Way ANOVA with Tukey correction. Differences between the two age groups were analyzed using Student's t-test (****$p < 0.0001$). Mice that were diabetic at the time of islet harvest are depicted in red (panels B–E). (Data reproduced directly from Osum et al. Sci Rep. 2018 May 29;8(1):8295.)

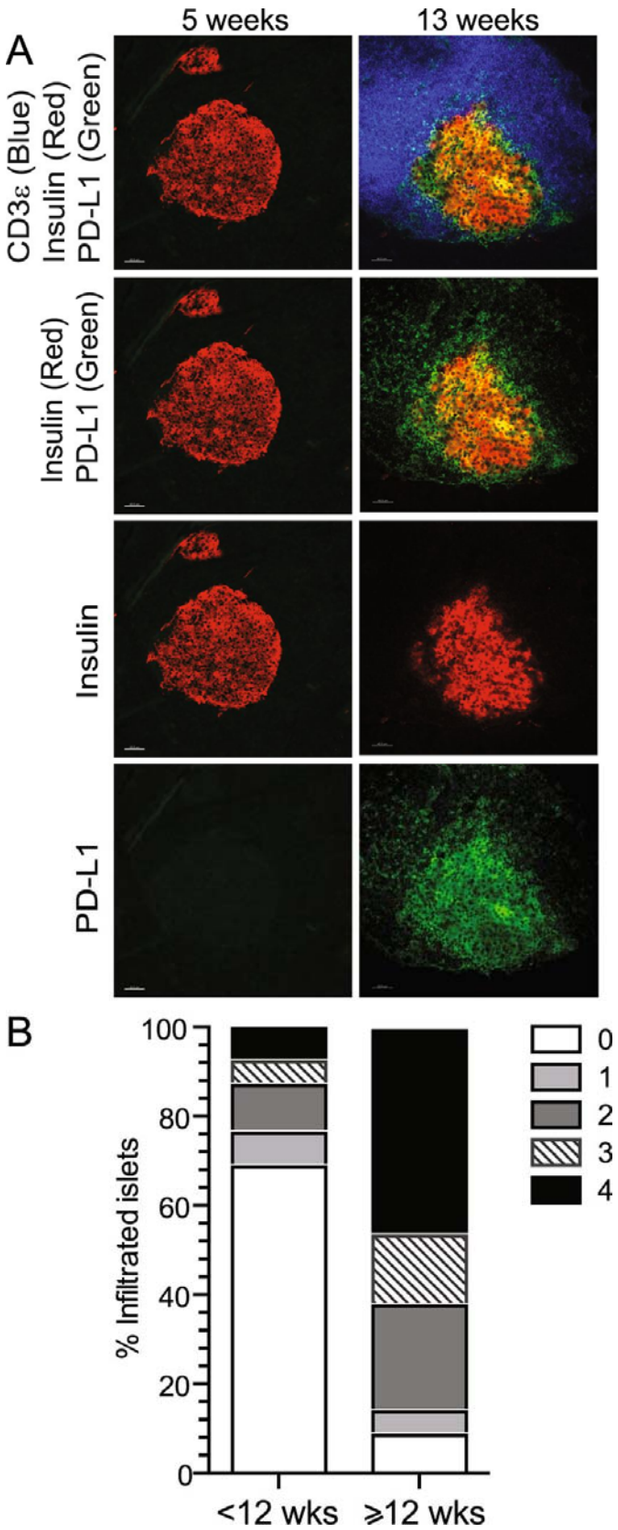

Fig. 4. PD-L1 expression is concurrent with T cell infiltration within pancreatic islets in NOD mice. (**A**) PD-L1 expression and T cell infiltration in pancreatic islets from 5 and 13 week old NOD mice. Scale bar corresponds to 40 µm. (**B**) Cumulative insulitis scores from younger (<12 week) and older (≥12 week) NOD mice. Data represent >80 islets per age group, compiled from at least 4 independent experiments with 3–14 mice/experiment. (Data reproduced directly from Osum et al. Sci Rep. 2018 May 29;8(1):8295.)

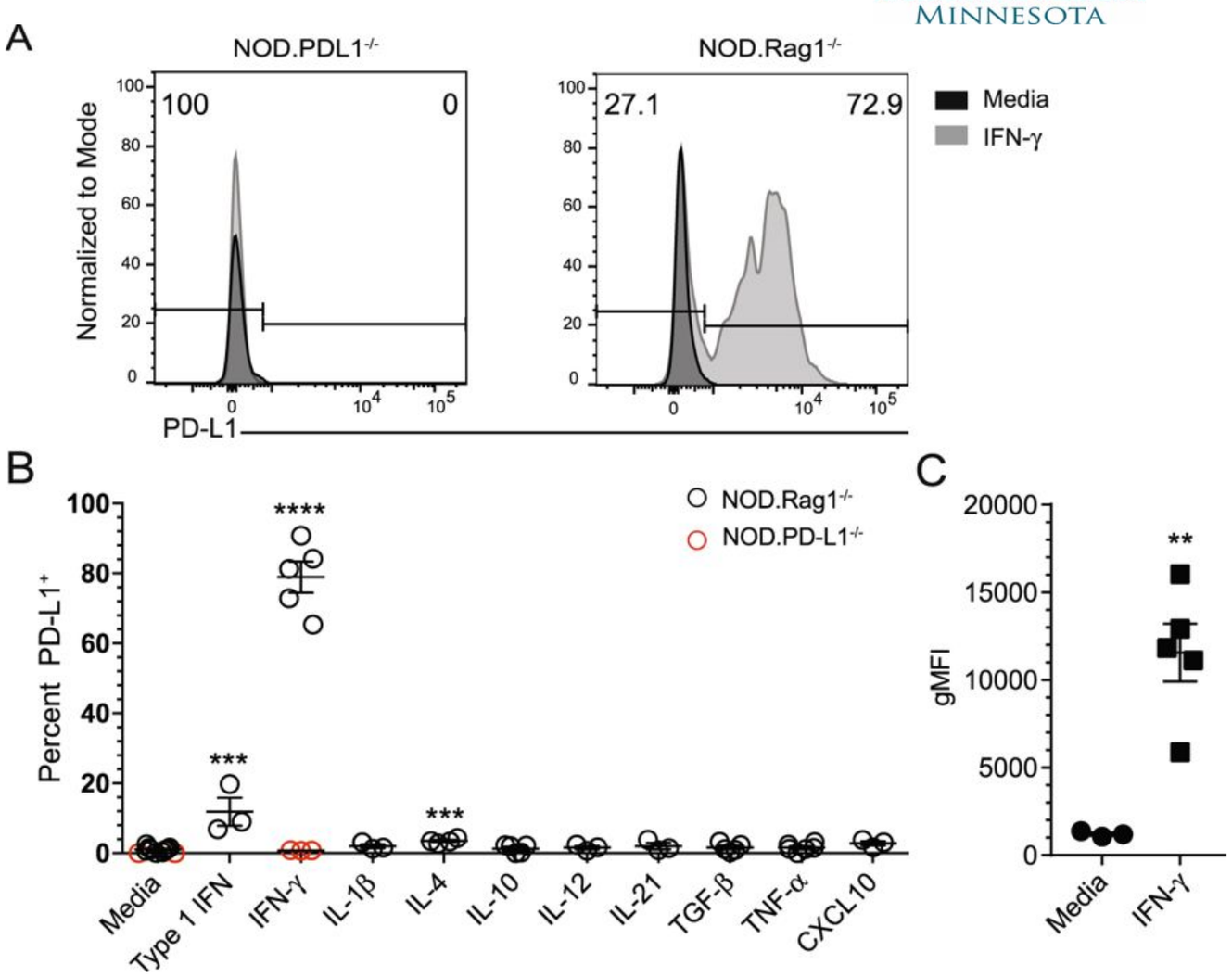

Figure 5. IFN-γ increases PD-L1 expression on mouse β cells. (**A**) Representative histogram plots of PD-L1 expression from pancreatic islets isolated from (left panel) NOD.Rag1$^{-/-}$PD-L1$^{-/-}$ negative control and (right panel) NOD.Rag1$^{-/-}$ mice were cultured overnight with media alone or IFN-γ. (**B**) Frequency of PD-L1^{+} β cells isolated from NOD.Rag1$^{-/-}$ (black) or NOD.PD-L1$^{-/-}$mice (red) after overnight culture with media alone or cytokines as indicated. (**C**) PD-L1 geometric mean fluorescence intensity (gMFI) of NOD.Rag1$^{-/-}$ β cells cultured with media alone or IFN-γ from (**B**). Data are representative from 2–4 independent experiments and 3–5 mice/group for each experiment. The mean is shown with a black line ± standard deviation. Significance was determined by Student's t-test. **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to media alone. (Data reproduced directly from Osum et al. Sci Rep. 2018 May 29;8(1):8295.)

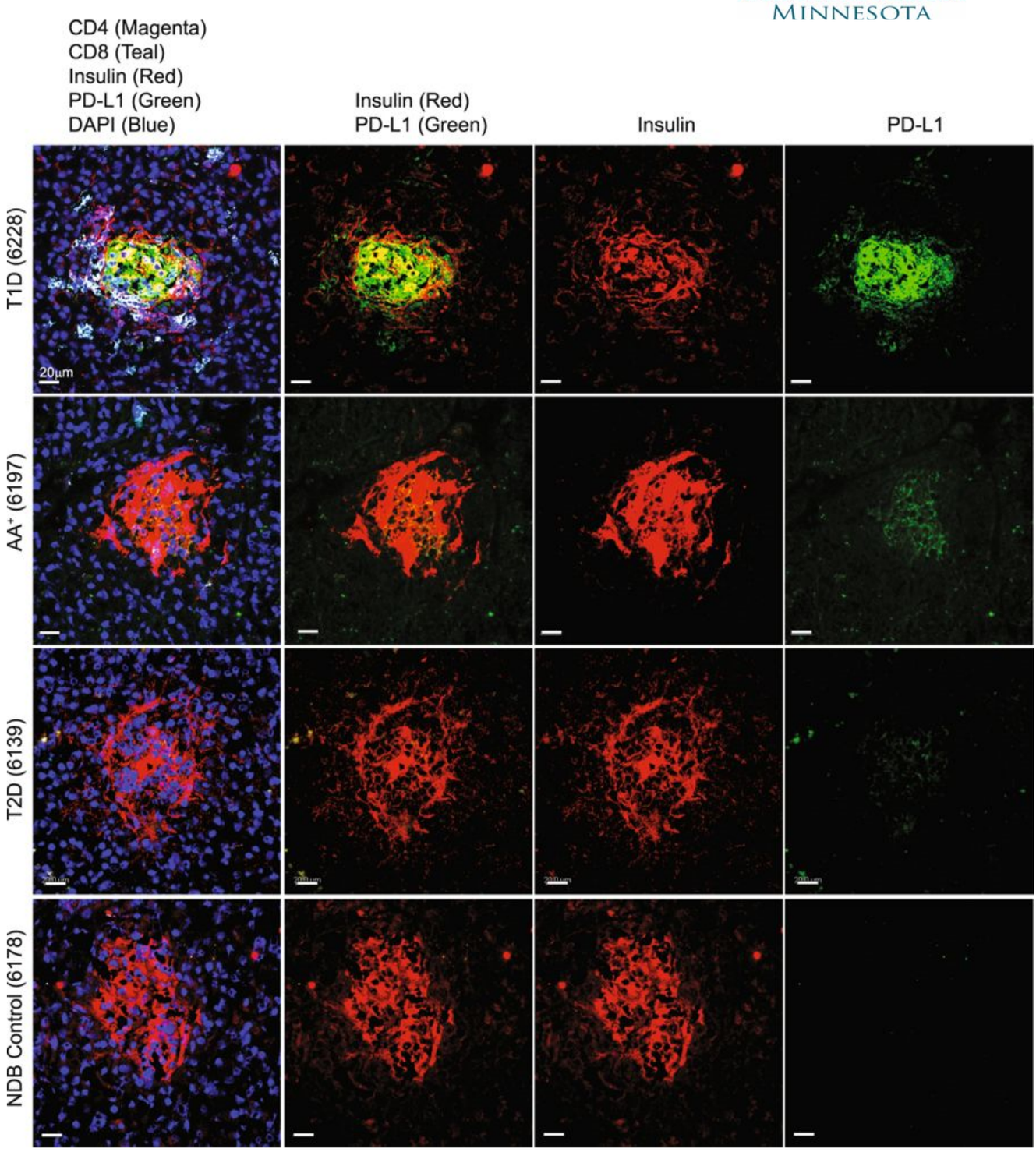

Figure.6. Human pancreas tissue from type 1 diabetic subjects express PD-L1. Human pancreas sections from type 1 diabetic (T1D), autoantibody positive (AA^+), type 2 diabetic (T2D), and non-diabetic controls (NDB) were obtained from the Network for Pancreatic Organ Donation (nPOD) and stained for T cell markers (CD4, CD8), insulin, and PD-L1. Shown are representative islets from each group with 7–15 unique islets analyzed from three independent experiments with one patient per group. Scale bar corresponds to 20 µm. (Data reproduced directly from Osum et al. Sci Rep. 2018 May 29;8(1):8295.)

Please list any of the following that have resulted from the Minnesota Regenerative Medicine grant funding:
Publications and/or manuscripts submitted for publication:

1. Spanier JA, Sahli NL, Wilson JC, Martinov T, Dileepan T, Burrack AL, Finger EB, Blazar BR, Michels AW, Moran A, Jenkins MK, **Fife BT**. Increased Effector Memory Insulin-Specific CD4+ T Cells Correlate With Insulin Autoantibodies in Patients With Recent-Onset Type 1 Diabetes. *Diabetes*. 2017 Dec;66(12):3051-3060.

Abstract
Type 1 diabetes (T1D) results from T cell-mediated destruction of insulin-producing β-cells. Insulin represents a key self-antigen in disease pathogenesis, as recent studies identified proinsulin-responding T cells from inflamed pancreatic islets of organ donors with recent-onset T1D. These cells respond to an insulin B-chain (InsB) epitope presented by the HLA-DQ8 molecule associated with high T1D risk. Understanding insulin-specific T-cell frequency and phenotype in peripheral blood is now critical. We constructed fluorescent InsB10-23:DQ8 tetramers, stained peripheral blood lymphocytes directly ex vivo, and show DQ8+ patients with T1D have increased tetramer+ CD4+ T cells compared with HLA-matched control subjects without diabetes. Patients with a shorter disease duration had higher frequencies of insulin-reactive CD4+ T cells, with most of these cells being antigen experienced. We also demonstrate that the number of insulin tetramer+ effector memory cells is directly correlated with insulin antibody titers, suggesting insulin-specific T- and B-cell interactions. Notably, one of four control subjects with tetramer+ cells was a first-degree relative who had insulin-specific cells with an effector memory phenotype, potentially representing an early marker of T-cell autoimmunity. Our results suggest that studying InsB10-23:DQ8 reactive T-cell frequency and phenotype may provide a biomarker of disease activity in patients with T1D and those at risk.

2. Osum KC, Burrack AL, Martinov T, Sahli NL, Mitchell JS, Tucker CG, Pauken KE, Papas K, Appakalai B, Spanier JA, **Fife BT**. Interferon-gamma drives programmed death-ligand 1 expression on islet β cells to limit T cell function during autoimmune diabetes. *Sci Rep*. 2018 May 29;8(1):8295. doi: 10.1038/s41598-018-26471-9.

Abstract
Type 1 diabetes is caused by autoreactive T cell-mediated β cell destruction. Even though co-inhibitory receptor programmed death-1 (PD-1) restrains autoimmunity, the expression and regulation of its cognate ligands on β cell remains unknown. Here, we interrogated β cell-intrinsic programmed death ligand-1 (PD-L1) expression in mouse and human islets. We measured a significant increase in the level of PD-L1 surface expression and the frequency of PD-L1+ β cells as non-obese diabetic (NOD) mice aged and developed diabetes. Increased β cell PD-L1 expression was dependent on T cell infiltration, as β cells from Rag1-deficient mice lacked PD-L1. Using Rag1-deficient NOD mouse islets, we determined that IFN-γ promotes β cell PD-L1 expression. We performed analogous experiments using human samples, and found a significant increase in β cell PD-L1 expression in type 1 diabetic samples compared to type 2 diabetic, autoantibody positive, and non-diabetic samples. Among type 1 diabetic samples, β cell PD-L1 expression correlated with insulitis. In vitro experiments with human islets from non-diabetic individuals showed that IFN-γ promoted β cell PD-L1 expression. These results suggest that insulin-producing β cells respond to pancreatic inflammation and IFN-γ production by upregulating PD-L1 expression to limit self-reactive T cells.

Disclosures/patents:
None

Reporting to all Minnesotans:
Briefly and using lay language, please describe your overall progress and how it is significant to the patients in need of regenerative medicine therapies in Minnesota. This will be used on the RMM website to demonstrate how funds are being used to advance the health of all Minnesotans.

We have generated a unique biomarker reagent and successfully determined and tracked these antigen-specific T cells in patients with T1D and compared these findings to non-diabetic control patients. We have observed an increase in antigen specific T cells during Type 1 diabetes (T1D). Success from this study could dramatically impact clinical care and revolutionize how we diagnose and treat autoimmune diabetes in Minnesota. The first major advancement will be earlier diagnosis and better insulin management to preserve remaining beta cells. Current biomarkers for T1D measure autoantibody titers. Their production occurs late in the disease process. Our tetramer reagents could be used to detect activated CD4 T cells before autoantibodies appear and pancreatic damage has occurred. Secondly, having a tool to track the number and activation of autoreactive T cells could be used in the clinic to identify disease progression or treatment efficacy. Future work for this project will apply this knowledge to develop novel approaches to limit the immune system as a treatment for self-reactive cells.