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SEXUAL DIMORPHISM IN THE PANCREATIC ISLET RESPONSE TO INFLAMMATION
AND AUTOIMMUNITY

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ABSTRACT

Type 1 diabetes (T1D) is a disease of insulin deficiency resulting from autoimmune destruction of the pancreatic β cells. Over a century after the discovery of insulin and its life-saving therapeutic utility, our understanding of who is at risk for T1D, what triggers disease onset, and how to prevent it remain limited. Increasing evidence implicates dysfunction of β cells themselves- including inflammatory signaling, endoplasmic reticulum stress, and neoantigen formation and presentation- in their targeting by autoreactive immune cells.

In this dissertation, I make use of the observation that T1D occurs more frequently in males after puberty and interrogate the role of sex in shaping β cell response to inflammation, a potential trigger for T1D onset. I hypothesized that female β cells may be preconditioned to respond more adaptively to inflammatory challenges and thus may be protected from developing more immunogenic phenotypes. I first challenged human islets from both male and female donors with proinflammatory cytokines and compared their responses using bulk RNA sequencing and mass spectrometry-based proteomics. To determine whether sexual dimorphism in islet inflammatory response may be driven by exposure to sex hormones, I utilized *in vitro* and *in vivo* treatments with 17 β -estradiol to study its influence on the β cell's transcriptome, proteome, and vulnerability to autoimmune diabetes. I provide evidence that human islets from male donors exhibit an exacerbated proinflammatory response to an identical inflammatory stimulus compared to islets from female donors, and I demonstrate that 17 β -estradiol suppresses diabetogenic inflammatory signaling and antigen presentation at the β cell, protecting against autoimmune diabetes in the non-obese diabetic model of T1D. Overall, the data presented in this dissertation support the notion that sex differences in β cell response to inflammation could contribute to the male predominance of- and relative female protection from-T1D.

To all my loved ones, to my many mentors along the way, and to my younger self.

Your unwavering belief that I could do this carried me through.

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LIST OF ABBREVIATIONS

AAB: Autoantibody	CD163/20/25/28/4/8: Cluster of
Actb: Beta-actin	differentiation 163/20/25/28/4/8
ADP: Adenosine disphosphate	CGM: Continuous glucose monitoring
AGR2: Anterior gradient 2	CHOP: C/EBP Homologous Protein
ATACseq: Assay for transposase-accessible	CO₂: Carbon dioxide
chromatin using sequencing	COL3A1: Collagen type III alpha 1 chain
ATP: Adenosine triphosphate	CTLA4: Cytotoxic T-lymphocyte associated
B2m: Beta-2-microglobulin	protein 4
BACH2: BTB Domain and CNC Homolog 2	CXCL2/3/10: C-X-C motif chemokine ligand
Bcl-2: B-cell leukemia/lymphoma protein 2	2/3/10
BCR: B-cell receptor	DAB: 3,3'-diaminobenzidine
BDC2.5: Beta-cell derived clone 2.5	DAMP: Damage-associated molecular pattern
BiP: Binding immunoglobulin protein	DEG: Differentially expressed gene
BMI: Body mass index	DKA: Diabetic ketoacidosis
BMP: Bone morphogenic protein	DMEM: Dulbecco's Modified Eagle Medium
BSA: Bovine serum albumin	DNA: Deoxyribonucleic acid
Bst2: Bone marrow stromal cell antigen 2	DPP4: Dipeptidyl peptidase 4
C-MAF: Cellular musculoaponeurotic	DRP1: Dynamin-related protein 1
fibrosarcoma	E1: Estrone
Casp3: Cysteine-aspartic acid protease 3	E2: 17 beta-Estradiol
Casp8: Cysteine-aspartic acid protease 8	E3: Estriol
	eIF2α: Eukaryotic initiation factor 2 alpha

ELISA: Enzyme-linked immunoassay	Greb1: Growth regulating estrogen binding 1
EMT: Epithelial-mesenchymal transition	GSEA: Gene set enrichment analysis
ER: Endoplasmic reticulum	Gzma: Granzyme A
ERAD: Endoplasmic reticulum-associated protein degradation	Gzmb: Granzyme B
ERα/β: Estrogen receptor alpha/beta	Gzmm: Granzyme M
ERE: Estrogen response element	Gzmk: Granzyme K
Esr1: Estrogen receptor 1	H2-K1: Histocompatibility 2, K1 region
FC: Fold-change	HbA1c: Glycated hemoglobin
FDA: Food and Drug Administration	HBSS: Hank's balanced salt solution
FGF: Fibroblast growth factor	HCl: Hydrochloric acid
Fol2r: Folate receptor beta	Hes1: Hairy and enhancer of split-1
FOXO1: Forkhead box protein O1	HIP: Hybrid insulin peptide
Foxp3: Forkhead box protein 3	HLA: Human leukocyte antigen
G6P: Glucose-6-phosphate	HPAP: Human Pancreatic Analysis Program
GAD65: Glutamic acid decarboxylase 65	IA-2: Insulinoma-associated protein 2
GATA4: GATA-binding factor 4	IFIH1: Interferon induced with helicase domain 1
GCK: Glucokinase GLP-1: Glucagon-like peptide 1	IFN-α: Interferon alpha
GLUT2/4: Glucose transporter 2/4	IFN-γ: Inteferon gamma
GPER1: G-protein-coupled estrogen receptor 1	IGFBP4: Insulin like growth factor binding protein 4
GPR94: Glucose-related protein 94	IIDP: Integrated islet distribution program
	IL-1β: Interleukin 1 beta

IL-2/6/15: Interleukin 2/6/15	Mrc1: Mannose receptor C type 1
IL2RA: Interleukin 2 receptor subunit alpha	MS: Multiple sclerosis
iNKT: Invariant natural killer T cell	NaCl: Sodium chloride
INS: Insulin	NES: Normalized enrichment score
INSM1: Insulinoma-associated 1	NF-κB: Nuclear factor kappa beta
Irf1: Interferon regulatory factor 1	NFE2L2/NRF2: Nuclear factor erythroid 2-related factor 2
ISG15: Inteferon-stimulated gene 15	NGN3: Neurogenin 3
ISL1: ISL lim homeobox 1	NK: Natural killer
JAK/STAT: Janus kinase/signal transducers and activators of transcription	NKX6.1: NK6 Homeobox 1
LAMA2: Laminin subunit alpha 2	NOD: Non-obese diabetic
LDHA: Lactate dehydrogenase A	NOD-SCID: Non-obsese diabetic severe combined immunodeficiency
LH: Lutenizing hormone	Ovol2: Ovo Like Zinc Finger 2
MAFA: Maf BZIP translation factor A	PC1/2/3: Prohormone convertase 1/2/3
MCT-1: Monocarboxylate transporter 1	PCR: Polymerase chain reaction
MDA-5: Melanoma differentiation associated protein 5	PD-1: Programmed cell death protein 1
MDS: Multi-dimensional scaling	pDC: Plasmacytoid dendritic cell
MFN1/2: Mitofusin ½	PDGFRA: Platelet-derived growth factor receptor alpha
MHC: Major histocompatibility complex	PDX1: Pancreatic duodenal homeobox 1
MIN6: Mouse insulinoma 6	Pgr: Progesterone receptor
MODY4: Maturity onset diabetes of the young 4	PIC: Proinflammatory cytokines

Prf1 : Perforin 1	STAT1/2 : Signal transducer and activator of transcription 1/2
PRR : Pattern recognition receptor	
PTPN2/22 : Protein tyrosine phosphatase non-receptor type 2/22	STZ : Streptozotocin
REL : Reticuloendotheliosis	T1D : Type 1 diabetes
RNA : Ribonucleic acid	T2D : Type 2 diabetes
ROS : Reactive oxygen species	TCA : Tricarboxylic acid
RYR2 : Ryanodine receptor 2	Tcf4 : Transcription factor 4
scRNA-seq : Single cell RNA sequencing	TCR : T cell receptor
SERCA2B : sarcoplasmic reticulum calcium ATPase 2B	TEDDY : The Environmental Determinants of Diabetes in the Young
SERPINA3 : Serine protease inhibitor A3	TFAP2A/C : Transcription factor AP-2 alpha/gamma
SGLT2 : Sodium-glucose cotransporter 2	TGF-β : Transforming growth factor beta
SHH : Sonic hedgehog	TGFB2 : Transforming growth factor beta 2
Siglech : Sialic acid binding Ig-like lectin H	Th1/2/17 : Type 1/2/17 helper T cell
SLC2A2 : Solute carrier family 2, facilitated glucose transporter member 2	TIMP3 : Tissue inhibitor of metalloproteinase 3
SLE : Systemic lupus erythematosus	TLR2/7/8 : Toll-like receptor 2/7/8
SOD : Superoxide dismutase	TNF-α : Tumor necrosis factor alpha
SOX9 : SRY-Box Transcription Factor 9	TRAIL : Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
SPARC : Secreted protein, acidic and rich in cysteine	Treg : Regulatory T cell
	TSIX : anti-X-inactive specific transcript

TTY15: Testis Expressed Transcript, Y-

Linked 15

UCN3: Urocortin 3

UMI: Unique molecular identifier

UPR: Unfolded protein response

UTY: Ubiquitously Transcribed

Tetratricopeptide Repeat Containing, Y-

Linked

Veh: Vehicle

Wnt: Wingless-related integration site

XIST: X-inactive specific transcript

ZFY: Zinc finger Y-chromosomal protein

ZnT8: Zinc transporter 8

CHAPTER 1: INTRODUCTION

Healers and scientists have observed the syndromes of intense thirst, excessive sugary urine, and emaciation that we now know as diabetes mellitus for millennia^{1,2}. Before the discovery of insulin just a century ago, diabetes had a grim prognosis- as estimated 90% of those diagnosed died within 5 years³⁻⁵. With careful management aided by blood glucose monitoring and insulin treatment, people now routinely live with diabetes decades beyond diagnosis. This drastic improvement drew upon centuries of scholarship relating diabetes to composition of the diet, to the function of the pancreas, and then specifically to the secretions of the islets of Langerhans. Today, we know that diabetes pathophysiology involves a complex dialog between the cells of the pancreas, the immune system, peripheral tissues, and the genetic and environmental factors that influence their functions. Even so, the various forms of diabetes we now recognize remain life-altering diagnoses requiring daily maintenance with significant complications, limited prevention strategies, and no known cures. In this dissertation, I leverage the observation that diabetes occurs less frequently in females, investigating how the sex hormone estradiol influences the response of the β cell- the insulin producer of the pancreatic islet- to inflammatory stress, a key precipitator of autoimmune (Type 1) diabetes.

1.1 The Pancreatic Islet

The pancreas is a comma-shaped organ in the upper abdomen, nestled between the stomach and the small intestine. The majority of the pancreas is comprised of exocrine cells organized into lobular clusters ('acini'), which manufacture protein-, carbohydrate-, and fat-digesting enzymes delivered into the duodenum through the pancreatic ducts. Interspersed among the many acini and making up only 1-2% of the pancreas are the 'islets of Langerhans,'⁶ spherical formations of endocrine cells responsible for the secretion of the metabolic hormones

insulin (β cells), glucagon (α cells), somatostatin (δ cells), ghrelin (ϵ cells), and pancreatic polypeptide (PP cells) into the bloodstream. Thus, the exocrine pancreas facilitates the release of nutrients from food as it traverses the intestine, while the endocrine pancreas cues the cells of the body to uptake and either store or metabolize those nutrients for energy to maintain metabolic homeostasis. In this section, I will briefly review the development of the pancreas, the role of pancreatic hormones in glucose homeostasis, and the defining features of the insulin-producing β cell.

1.1.1 Pancreatic Development

About 3 weeks post-fertilization, the human embryo undergoes gastrulation, the process by which a single layer of totipotent cells transforms into 3 distinct layers of multipotent cells—the ectoderm, which will become the skin and nervous system, the mesoderm, which will become the cardiovascular system, musculoskeletal system, and connective tissue, and the endoderm, which will form the lungs and the gastrointestinal tract. Around gestational week 4 (Gw4), the pancreas begins to emerge from the foregut endoderm. Appearing first is a dorsal bud, which will become the tail, body, and upper head of the pancreas, followed several days later by the ventral bud, which will migrate to fuse with the dorsal bud and form the inferior part of the pancreatic head. The pancreatic buds are distinguished from the surrounding foregut endoderm by excluding expression of morphogenic factor Sonic hedgehog (SHH), and by allowing expression of transcription factors pancreatic and duodenal homeobox 1 (PDX1), SRY-box 9 (SOX9), and GATA-binding protein 4 (GATA4). From there, expression levels of a series of transcription factors determine cell type fates in the developing pancreas. Acinar cell progenitors ('tip' cells), for example, maintain high expression of GATA4, while GATA4 declines in the 'trunk' cells that precede ductal and endocrine cells⁷. Of the 'trunk' cells, those that maintain SOX9 become ductal, while low SOX9 and a transient wave of neurogenin 3

(NGN3) expression mark the emerging endocrine cells- insulin positive β cells (Gw~7.5), followed by α and δ cells (Gw8), then PP and ϵ cells (Gw9)^{7,8}.

There is some debate as to how endocrine cells sort into islets during development. The early pancreas contains an epithelial mass embedded in a loose mesenchyme which secretes developmental signals like fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), transforming growth factor β (TGF- β), Notch ligands, and WNTs that determine the balance between endocrine and exocrine fates.⁹ Whether endocrine cells fully differentiate before or after separating from the remainder of the epithelium ('delamination'), and whether they delaminate as individual cells or as established clusters, are disputed. Mature islets in mice and other model species are arranged by cell type, featuring a β cell core encircled by an outer mantle of α , δ , PP, and ϵ cells. Interestingly, while human islets pass through a stage of similar compartmentalization during development, the mature human islet is a mosaic of all 5 cell types (**Figure 1**)^{10,11}. In the adult human islet, β cells are most abundant (~60%), followed by α cells(~30%) δ , PP, & ϵ cells (<10%).¹²

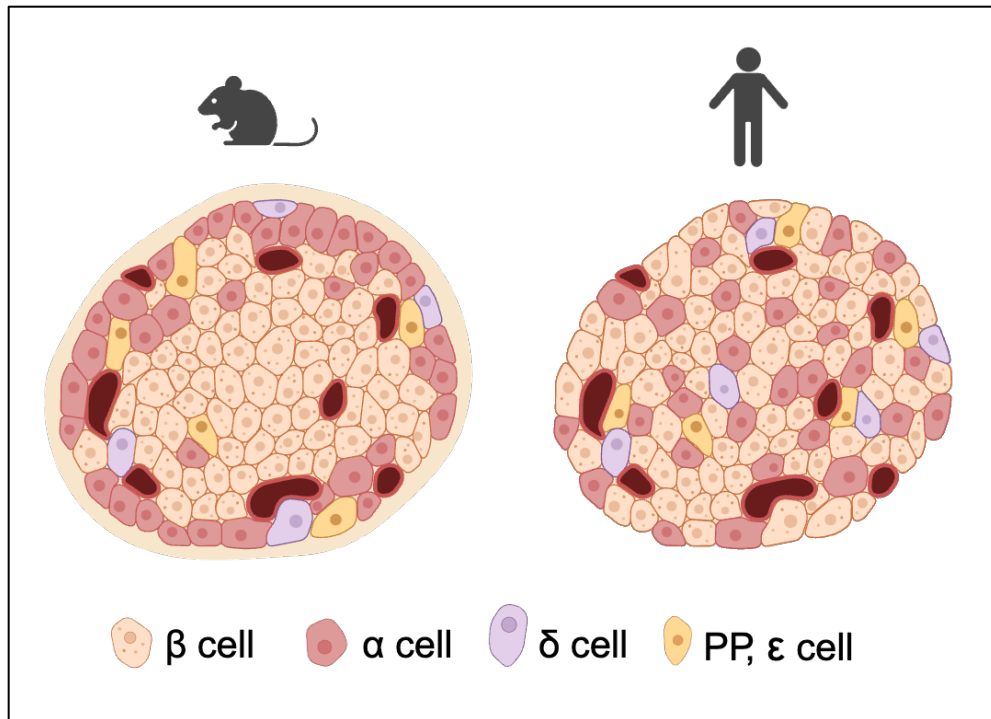


Figure 1: Organization of murine and human pancreatic islets. Created with BioRender.com.

1.1.2 Pancreatic Hormones and Glucose Homeostasis

The human body works fastidiously to maintain blood glucose levels within a narrow range (about 4-6 millimolar during fasting), allowing our cells sufficient access to energy while avoiding the damaging effects of excess. Hypoglycemia is an imminent threat to health because some essential tissues, such as the central nervous system, rely almost entirely on circulating glucose for fuel, and without it they begin to fail¹³. Hyperglycemia, on the other hand, damages blood vessels and organs slowly over time through mechanisms including protein modification (e.g. glycation events that lead to ‘sticky’ blood cells), oversaturation of canonical glucose metabolism pathways, and activation of alternative metabolic pathways (e.g. polyol/sorbitol pathway), resulting in oxidative stress, endoplasmic reticulum (ER) stress, and inflammation¹⁴. This delicate balance relies heavily on pancreatic islet hormones.

Insulin, synthesized and secreted by the β cell, is the major hormone responsible for lowering fed-state blood glucose back to baseline. Insulin receptors are expressed across many tissues, including the brain, but the best studied ‘insulin-sensitive’ tissues are the skeletal muscle, white adipose tissue, and the liver. When insulin binds to its receptor, it sets off a signaling cascade that culminates in anabolic processes- moving glucose transporter GLUT4 from the cytosol to the plasma membrane to increase glucose influx in muscle and adipose tissue, stimulating glucose storage as glycogen in the muscle and liver, and suppressing hepatic gluconeogenesis and adipocyte lipolysis¹⁵. Glucagon, conversely, acts to mobilize stores of energy (i.e. via gluconeogenesis at the liver and kidneys¹⁶) in the fasting state, serving as a crucial counter regulator to insulin and a line of defense against hypoglycemia.

Islet cells must synthesize information not only from nutrient levels in the blood, but also from gut-derived incretins, sympathetic and parasympathetic innervation, and paracrine signals from one another to modulate their output of metabolic hormones¹⁷. After a meal, for example,

macronutrients stimulate L-cells of the gut epithelium to release glucagon-like peptide 1 (GLP-1), which binds to its receptor on the β cell and enhances glucose stimulated insulin secretion. When the β cell releases insulin, it also releases the peptide urocortin 3 (UCN3), which activates somatostatin release from the δ cell. Somatostatin then acts locally to inhibit secretion of both insulin and glucagon¹⁸. Unique adaptations prepare each islet endocrine cell type for its role in these life-sustaining feedback loops.

1.1.3 Insulin Production and Secretion

The human insulin gene (*INS*), which is transcribed almost exclusively in islet β cells, encodes the 110 amino acid preproinsulin peptide. Translation of the *INS* mRNA begins at a ribosome in the cytosol, and translation of the N-terminal signal peptide directs movement of the whole ribosome-mRNA complex to the ER. The signal peptide is cleaved in the ER lumen, resulting in the proinsulin peptide. Proinsulin folding is aided by ER lumen chaperones and protein disulfide isomerases; disulfide bonds form linking residues near the N terminus (B chain) to those near the C terminus (A chain). On its way through the trans-Golgi network, proinsulin is sorted into immature secretory granules containing proteolytic enzymes prohormone convertase 1/3 and 2 (PC1/3 and PC2). These enzymes cleave the linked A and B chains (mature insulin) from 'C peptide', the 31 amino acids between them in the proinsulin chain. Mature insulin crystallizes into hexamers in the presence of zinc ions in the secretory granules, while C-peptide remains soluble. Mature secretory granules are then ready to fuse with the plasma membrane and release insulin via exocytosis upon the appropriate signal.

At high blood glucose concentrations, glucose entering the β cell through facilitative glucose transporter GLUT2 is phosphorylated by glucokinase, forming glucose-6-phosphate (G6P). This is the rate-limiting step for glucose metabolism and is irreversible, effectively 'trapping' the glucose inside the β cell. Glucokinase is a low-affinity version ($K_m \sim 8\text{-}10\text{mM}$) of

the hexokinases found in other cells, ensuring that the glucose metabolism cascade that culminates in insulin secretion only happens when blood glucose is high. G6P then enters the glycolytic and oxidative phosphorylation pathways, generating ATP and raising the ATP/ADP ratio in the cell. As a result, ATP-sensitive potassium channels at the plasma membrane close, depolarizing the cell and opening voltage-gated calcium channels. Ca^{2+} flows down its concentration gradient into the cell, triggering fusion of secretory granules with the plasma membrane and release of insulin via exocytosis to the bloodstream.

1.1.4 β Cell Identity¹⁹

Mature β cell function requires the ability to sense blood glucose levels quickly and accurately, the biosynthetic capacity to mass produce and process insulin, the dexterity to fine-tune the release of insulin, and coordination with other islet cells to avoid over- or under-responding to glucose flux. Significant effort to date has shed light on how ' β cell identity'- the repertoire of cellular tools that equip β cells for these tasks- is developed *in vivo* and how alterations in β cell identity may be a consequence or even cause of diabetes²⁰⁻²³.

β cell identity is often assessed through the production of β cell-enriched transcription factors such as PDX1, MAFA, FOXO1 and NKX6.1. Together, these transcription factors guide the differentiation and maturation of β cells and maintain mature β cell function in adulthood by regulating genes related to glucose sensing and transport (e.g., *SLC2A2*, *GCK*), mitochondrial function (e.g., *MFN1/2*, *DRP1*), calcium signaling (e.g., *RYR2*, *SERCA2b*), and insulin production (e.g., *MAFA*, *PDX1*, *INS1/2*)²⁴⁻²⁷. Absence of *Pdx1* during murine and human development, for example, leads to complete pancreatic agenesis^{28,29}, while β cell specific *Pdx1* depletion in adult mice results in loss of insulin expression, downregulation of *MafA*, and rapid onset hyperglycemia³⁰. Heterozygous mutations in *PDX1* in humans lead to a form of monogenic diabetes termed maturity-onset diabetes of the young 4 (MODY4)³¹.

β cell identity also relies upon the *repression* of other cellular programs, as shown through inducible knockouts of enriched transcription factors in mice. Ablation of *Pdx1* derepresses α cell transcription factor MafB in adult β cells, generating a phenotype closely resembling α cells³⁰, and Nkx6.1 deletion leads embryonic or adult β cells to co-express insulin and somatostatin³². At least 60 ‘disallowed’ genes have been identified in β cells^{33–35}. Many of these regulate glucose sensing and metabolism, processes which are coupled to insulin exocytosis in the β cell and therefore must be fine-tuned. For example, β cells repress the expression of high-affinity hexokinases in favor of low-affinity glucokinase to avoid insulin release at low blood glucose levels^{36,37}. Similarly, low monocarboxylate transporter 1 (MCT-1) expression prevents β cells from taking up circulating lactate or pyruvate (i.e. during exercise)^{38,39}, and low lactate dehydrogenase A (LDHA) prevents interconversion of these metabolites and their entry into the TCA cycle^{40,41}. Also among the β cell repressed genes are cell proliferation factors, such as *Pdgfra*, *c-Maf*, and *Igfbp4*³⁵, whose repression likely contributes to the largely quiescent state of mature β cells. Selective gene repression is controlled by inhibitory actions of β cell transcription factors^{30,42}, epigenetic modifications^{43,44}, and by microRNAs and long noncoding RNAs.^{45–47}

Importantly, β cell identity is neither uniform nor static. Single-cell RNA sequencing (scRNA-Seq) and electrophysiological studies reveal subpopulations of β cells that exhibit different abundances, transcriptional signatures, maturity levels, and glucose response dynamics even within the same islet⁴⁸. Disentangling the roles of these subpopulations, how they interact, and how they shift during diabetes pathogenesis may enable us to manipulate the state of β cell identity as a therapeutic avenue.

1.2 Overview of Type 1 Diabetes Pathogenesis

Diabetes mellitus is a disease of chronic hyperglycemia that impacts approximately 10% of the global adult population (~828 million adults) and rising as of 2022, doubled from the figure of about 5% (~198 million adults) in 1990⁴⁹. Diabetes is listed among the World Health Organization's ten leading causes of death worldwide⁵⁰, with vascular pathologies like nephropathy and heart disease historically the most common causes of diabetes-related mortality⁵¹. Even among populations in which disease management is improving and vascular disease burden is declining, diabetes has been linked to depression, liver disease, infections, and even several types of cancer⁵². Importantly, this disease is not a monolith- there are several known etiologies requiring different therapeutic approaches.

Diabetes can result from a deficiency in the body's ability to respond to insulin, termed 'insulin resistance', and/or by a deficiency of insulin itself. While some rare forms of diabetes are monogenic (e.g. heterozygous *PDX1* mutations in MODY4, leading to inadequate insulin secretion), most cases are thought to be polygenic and to involve the interaction of genetic susceptibility and environmental insults. In the 21st century, type 2 diabetes (T2D) is the most common form and is characterized by insulin resistance. The course of T2D is thought to involve overnutrition, compensatory expansion of β cell mass and hyperinsulinemia, and eventually hyperglycemia as insulin sensitivity declines in the peripheral tissues. Type 1 diabetes (T1D), the focus of this dissertation, is the major form of insulin deficient diabetes and is driven by autoimmune destruction of the pancreatic β cells.

1.2.1 T1D Presentation

T1D is commonly conceptualized as a childhood-onset disease, with peaks in incidence between 5 to 7 and between 10 to 14 years of age^{53,54}. Children often present with polyuria, polydipsia, fatigue, and weight loss, while about one-third present with the life-threatening

condition of diabetic ketoacidosis (DKA), a severe metabolic acidosis resulting from hepatic β oxidation of free fatty acids to produce energy in the absence of insulin. Half or more of new-onset T1D cases, however, are identified in adults⁵⁵. Adult T1D presentation is more heterogeneous, though generally less aggressive than childhood T1D. Some adults do not rely on exogenous insulin at onset, for example, leading up to half of adult-onset T1D cases to be initially misclassified as insulin-resistant T2D^{56,57}. Across age groups, T1D is not typically detected before evolving into symptomatic dysglycemia. Genetic studies, families with T1D, and animal models continue to build our understanding of who is at risk for T1D and how the preclinical phase of the disease unfolds.

1.2.2 Genetic Component of T1D

The widely accepted model of T1D pathogenesis begins with an individual at genetic risk for disease, followed by an undefined environmental trigger of β cell autoimmunity (Stage 1), progression to presymptomatic dysglycemia (Stage 2), and finally to the symptomatic dysglycemia in which most people present (Stage 3) (**Figure 2**). T1D runs in families via a non-Mendelian inheritance pattern. First degree relatives of an individual with T1D have about a 1 in 20 risk of developing the disease, 15 times higher than the 1 in 300 risk among the general population⁵⁸. The vast majority of T1D cases (~90%), however, occur in individuals with no known relative with T1D^{54,59}. Genome wide association studies (GWAS) have identified over 50 susceptibility loci for T1D^{60–63}. Notably, about 50% of genetic risk can be accounted for in the region encoding human leukocyte antigen (HLA), the complex responsible for presenting peptide antigens to T cells⁶⁰. The remaining risk loci, including the insulin gene (*INS*) and many genes regulating inflammation and apoptosis (e.g. *PTPN22*, *CTLA4*, *BACH2*) represent more incremental modifiers of risk for disease progression. Interestingly, even among people with the highest-risk HLA haplotypes (DR3-DQ2, DR4-DQ8), only about 5% will develop diabetes by

the age of 15^{64,65}. This makes clear that HLA variants are insufficient on their own to kickstart islet autoimmunity.

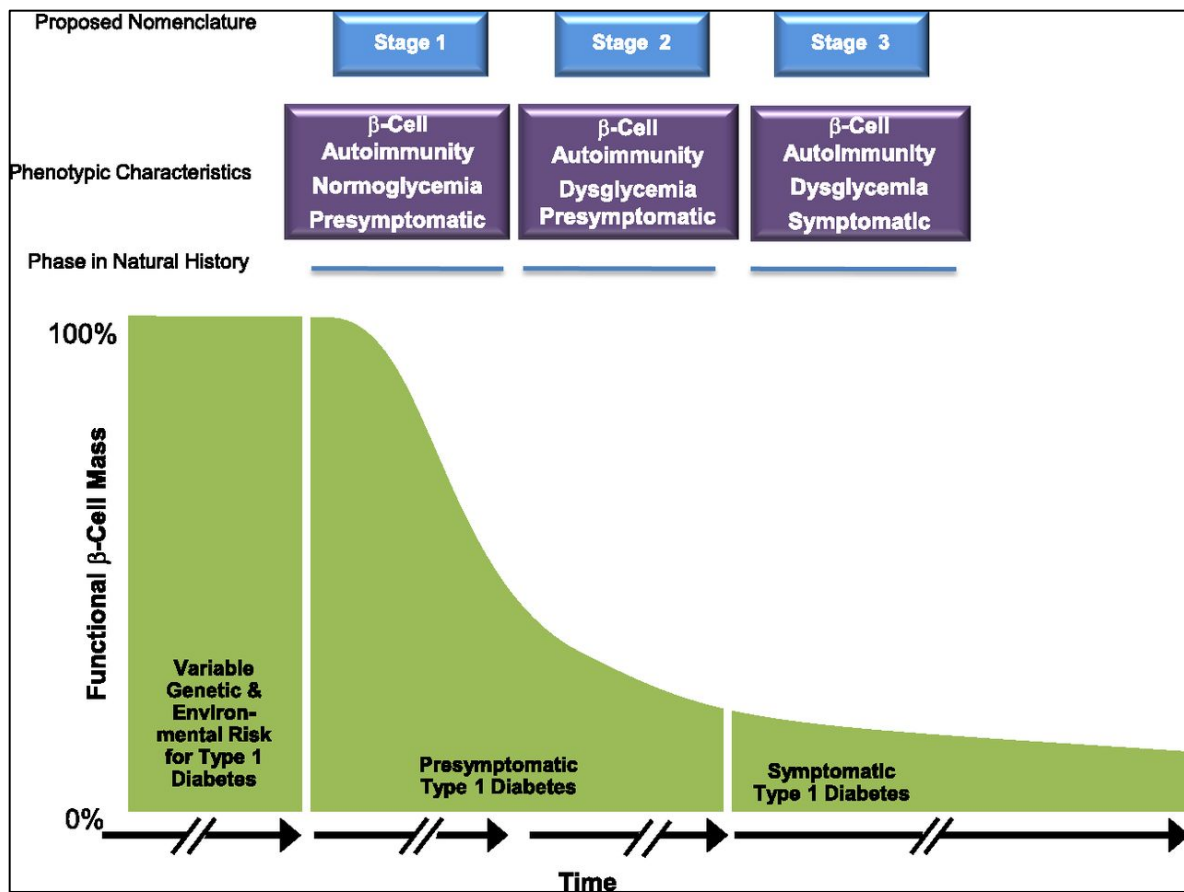


Figure 2: Stages of T1D natural history.

SOURCE: Insel et al, *Diabetes Care* (2015)⁶⁶. Open Access under terms of CC BY license.

1.2.3 Environmental Triggers of Autoantibody Development, T1D

The relatively low rate of T1D development in individuals with the most potent genetic risk factors, in combination with the climbing rate of T1D incidence globally, suggests that genetic risk must interact with some additional insult to trigger islet autoimmunity and precipitate T1D. Prospective cohort studies in people with T1D associated HLA haplotypes or first degree relatives with T1D, such as The Environmental Determinations of Diabetes in the Young (TEDDY)⁶⁷ and TrialNet's "Pathways to Prevention"⁶⁸, have been powerful tools in exploring the relationship between disease progression and environmental factors. TEDDY collected serial samples from participants over more than two decades, with primary endpoints being the development of autoantibodies directed against islet antigens (most commonly insulin, glutamic acid decarboxylase (GAD65), zinc transporter 8 (ZnT8A), or protein phosphatase like A2 (IA-2)) and the development of diabetes. Underscoring the importance of autoantibodies as a clinical milestone, an individual's risk of progressing to T1D increases with increasing numbers of different islet autoantibodies- 2 or more portend a 70-80% risk of T1D within 10 years and a lifetime risk approaching 100%^{66,69}. Data from TEDDY have supported associations between islet autoantibody development and early viral infections (particularly enteroviruses), gastroenteritis, and variable influences of dietary factors and maternal health⁷⁰. TEDDY has also shown associations between early age of seroconversion and progression from multiple autoantibody positivity to T1D⁷¹. The mechanisms by which these risk factors contribute to autoantibody development and disease progression are under active investigation.

1.2.4 T1D Management

The overall goal of T1D management is to avoid imminent threats like DKA and hypoglycemia and to decrease the likelihood of long-term complications of hyperglycemia (i.e. vascular disease)⁷². To achieve this, healthcare providers work with families to personalize

glucose monitoring and insulin treatment regimens, aiming to maintain an HbA1c level of around 7%. In the pancreas, insulin is stored in zinc-containing granules in an inactive hexameric form which must disassociate in the blood to become biologically active. Different pharmacological formulations of insulin manipulate multimer stability to create rapid-, intermediate-, or long-acting therapeutics that can be taken before meals, twice per day, or once per day, respectively. Safe insulin treatment must be combined with regular glucose monitoring. Integrated and automated tools are increasingly available; in 2016 the FDA approved the first combined continuous glucose monitor (CGM) and insulin delivery system, sometimes referred to as an “artificial pancreas”, which delivers basal insulin in accordance with glucose readings⁷³. No adjunctive therapies are currently recommended in T1D, though patients with additional signs of metabolic dysfunction (e.g. excess weight, insulin resistance) are sometimes prescribed GLP1 receptor agonists, inhibitors of sodium glucose cotransporter 2 (SGLT2) or dipeptidyl peptidase-4 (DPP4), or metformin with the goal of improving glycemic control and decreasing insulin requirements⁷⁴.

1.2.5 Innovations and Ongoing Challenges in T1D Management

The outlook for people with T1D has improved dramatically in the last 100 years. The ability to live a relatively healthy life for decades with a disease that was once uniformly fatal is perhaps one of the starkest achievements of modern medicine. Importantly, living well with T1D still requires diligent management and costly supplies for the remainder of one’s life. Studies report that those with T1D experience diminished health-related quality of life^{75,76} and decreased lifespans compared to nondiabetic peers⁷⁷. The field is actively pursuing strategies that might restore β cell function to those with T1D, allowing them to produce their own insulin. Allogenic human islet transplantation, in which cadaveric donor islets are separated from the exocrine pancreas and implanted into a recipient’s portal vein, has shown great promise in reducing

hyperglycemia^{78,79}. This approach is limited by the challenges of chronic immunosuppression⁸⁰, recurrence of islet autoimmunity in grafts⁸¹, and scarcity of donor tissues. Protocols are in development to differentiate functional β cells from induced pluripotent stem cells in culture as an alternative source, but strategies to avoid ongoing autoimmunity have not been established.

In the meantime, the field is also making significant efforts to improve prediction of T1D risk and develop interventions to prevent or slow the progression of β cell destruction. Autoantibody screening efforts have focused on family members of those with T1D, yet 90% of individuals presenting with T1D have no known relative with T1D^{54,59}. Generalized screening for T1D is under vigorous discussion in recent years^{82–84}. Advocates argue that broader screening could decrease the ~30% of new T1D cases presenting in life-threatening DKA⁸³, and that the use of features beyond just autoantibody number (i.e. type, titers) might inform more precise risk stratification⁸⁵. Medicine can now do more than just predict risk for those who screen multiple autoantibody positive. A single course of teplizumab, an immunosuppressant monoclonal antibody against the T cell co-receptor CD3, delayed progression to Stage 3 T1D by nearly 3 years in a randomized controlled trial and was approved by the FDA in 2022^{86,87}. While this is an incredible advance in T1D prevention, it must be noted that about half of individuals in the teplizumab group still progressed to Stage 3 within 5 years⁸⁷. We still have much to learn about the factors determining whether an at-risk person progresses, why some at-risk people don't respond to teplizumab, and the events that trigger β cell specific autoimmunity in the first place.

1.3 T1D Pathophysiology

1.3.1 The adaptive immune system and T1D development

In many regards, T1D fits the classical description of an organ-specific autoimmune disease, a condition in which T and B lymphocytes become reactive against 'normal' components of a particular organ. The importance of HLAs and other immune response genes like *PTPN22*

and *IL2RA* to the heritability of T1D, the characteristic development of islet-directed autoantibodies, and the observed pathological feature of lymphocytic infiltration of the islets ('insulinitis') make clear that the immune system plays a major role in T1D.

The ability of the immune system to distinguish foreign (i.e. dangerous) antigens from host-derived antigens is termed 'immune tolerance', and the organ-specific collapse of that tolerance across various autoimmune diseases is not well understood. During lymphocyte development, the genes encoding the antigen-binding portions of the T or B cell receptors (TCR or BCR) in each cell undergo a stochastic reorganization called V(D)J recombination that makes possible $>10^{60}$ different sequences and thus broad protection from pathogenic molecules we may encounter throughout life. Before T cells are released into circulation, these rearranged receptors go through a two-part vetting process in the thymus to ensure that they are effective in engaging with antigen-bearing major histocompatibility complexes (MHC; also referred to as HLA in humans) (positive selection), but not strongly reactive to self-antigens (negative selection). T cells that don't meet these criteria are subject to 'clonal deletion' via apoptosis or diversion to become Foxp3+ 'regulatory T cells', which act as antigen-specific immunosuppressors in the periphery. Failure of this 'central tolerance' process, and thus escape of autoreactive cells into the circulation, is thought to contribute to autoimmune disease. Strikingly, studies suggest that islet-directed autoreactive T cells circulate at similar levels in nondiabetic people and those with T1D, making up part of the normal T cell repertoire⁸⁸. These cells are, however, enriched in the pancreata of those with T1D, pointing to a difference in homing or peripheral regulation rather than a failure of central tolerance in T1D⁸⁸.

Because autoreactive T cells can persist after maturation in the thymus, the body also has mechanisms of 'peripheral tolerance' to diminish emergent self-reactivity in the tissues.

Activation of a T cell's effector function requires not only engagement of the TCR with a peptide-MHC complex, but also a co-stimulatory signal such as co-receptor CD28 on the T cell engaging with its ligand (**Figure 3**). If a TCR is engaged in the absence of a costimulatory signal, or in the presence of an inhibitory second signal like PD-1 or CTLA-4 engagement, the T cell can enter a state of hyporesponsiveness called 'anergy'. Regulatory T cells, or 'Tregs', are another important player in peripheral tolerance. Tregs bind to their target antigens primarily on MHC-II complexes, which are presented by 'professional' antigen presenting cells of the innate immune system like macrophages and dendritic cells. Upon antigen binding, Tregs enact their immunosuppressive functions on other immune cells through mechanisms including releasing inhibitory cytokines TGF β and IL-10, expressing inhibitory surface molecules like CTLA-4, or even through cytotoxicity of other effector cells. Individuals with T1D seem to have a comparable number of circulating Tregs (CD4⁺CD25⁺) to their nondiabetic counterparts⁸⁹⁻⁹¹, though with evidence of diminished expression of Treg transcription factor FOXP3 and weakened response to T cell growth and differentiation factor interleukin 2 (IL-2)^{92,93}. This is one possible mechanism of impaired peripheral tolerance in T1D.

B cells are thought to contribute to T1D development by presenting islet autoantigens to T helper cells, as well as through their production of islet autoantibodies and proinflammatory cytokines⁹⁴. B cell depletion with CD20 monoclonal antibody rituximab helped to maintain serum C-peptide levels in new-onset T1D⁹⁵. B cells are subject to central tolerance mechanisms in the bone marrow, as well as to peripheral tolerance mechanisms like anergy when they bind to self-antigen in the absence of costimulatory signals. T1D risk gene *PTPN22* encodes a phosphatase important to the maintenance of B cell anergy via suppression of inflammatory pathways like NF- κ B, suggesting that failure of B cell peripheral tolerance may contribute to

T1D. Interestingly, unlike in antibody-mediated autoimmune diseases like systemic lupus erythematosus (SLE) or Grave's Disease, there is a paucity of evidence for an actual pathogenic role of islet directed autoantibodies in T1D⁹⁶. In the non-obese diabetic (NOD) mouse model of T1D, impairing immunoglobulin secretion in B cells did not prevent or delay diabetes development, suggesting that B cell derived islet autoantibodies may be a side effect more so than a driver of the disease process⁹⁷.

Overall, there is compelling evidence for involvement of T and B lymphocytes in T1D pathophysiology. The available data suggests that self-tolerance breaks down not during TCR and BCR screening, but rather in the regulation of immune function in the periphery. Several fundamental questions, however, remain: what events initiate the presentation of islet self antigens to the adaptive immune system, and why are β cells in particular targeted while other islet cell types are spared?

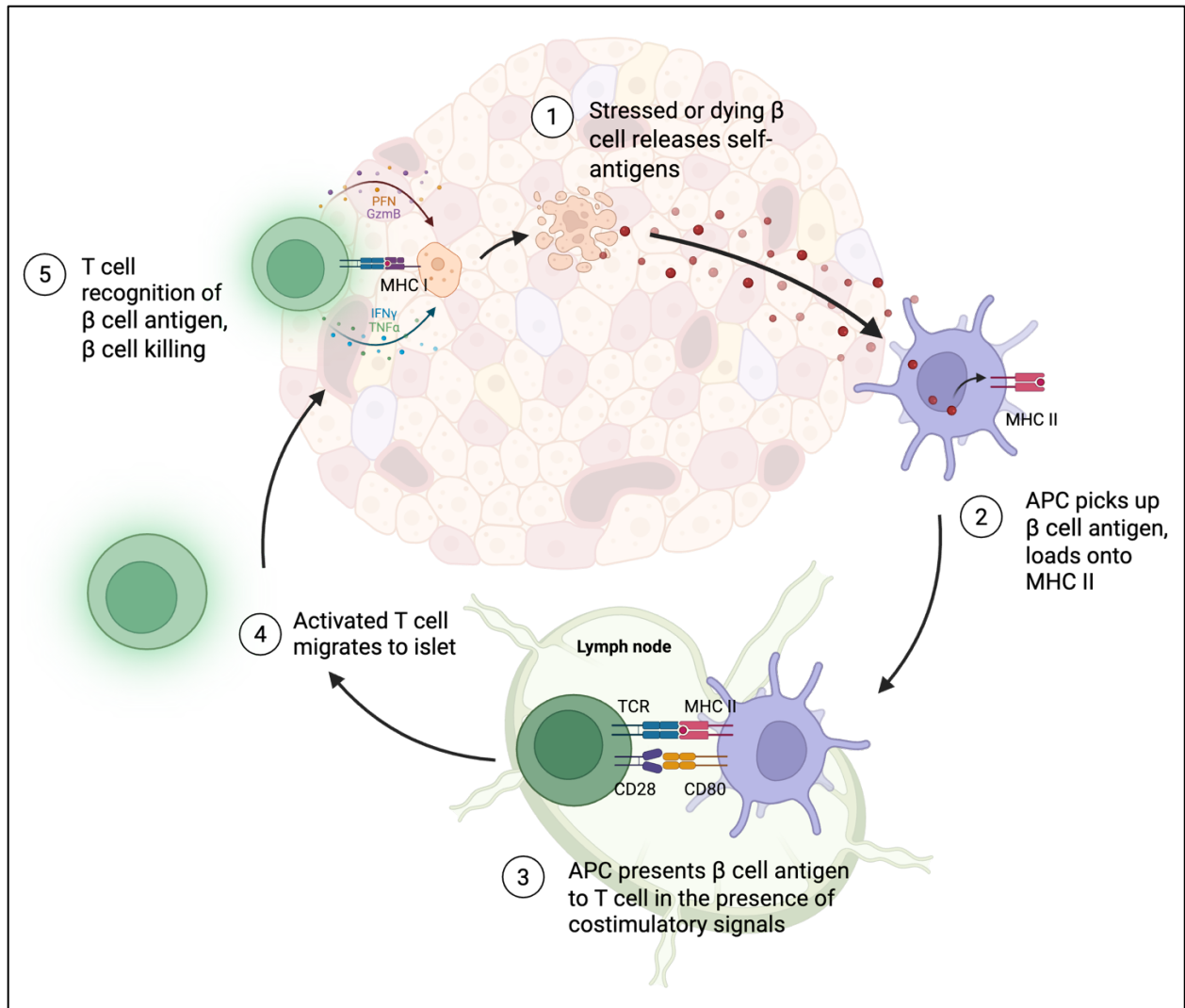


Figure 3: Initiation of β cell autoimmunity. Created with BioRender.com.

1.3.2 β cell stress precedes T1D

Autoimmune diseases are by and large understood as a failure of immune function, wherein overactive or misdirected immune cells destroy normal, healthy cells of the host. Mounting evidence suggests that dysfunction of the target organ may actually precede- perhaps even cause- the onset of autoimmunity (**Figure 4**) It was noted in 2012 that people with recent-onset T1D had about 26% lower pancreatic volume than their nondiabetic counterparts⁹⁸. While one might attribute this to the autoimmune destruction of the β cells, islets are only estimated to make up 1-2% of the pancreatic volume. Further studies found that even autoantibody negative first degree relatives of those with T1D had smaller pancreata than control subjects on average⁹⁹, suggesting that at-risk individuals may already start out with some pancreatic deficiencies. There are no safe and established methods for biopsy of the pancreas in living human subjects or for quantification of β cell mass, so it is difficult to determine whether their smaller pancreata also indicate lower starting β cell mass. Numerous lines of evidence in human T1D and in mouse models do, however, draw a connection between β cell stress and dysfunction and the activation of β cell directed autoimmunity. In other words, the ‘trigger’ of T1D onset may lie within the β cell themselves^{100–104}.

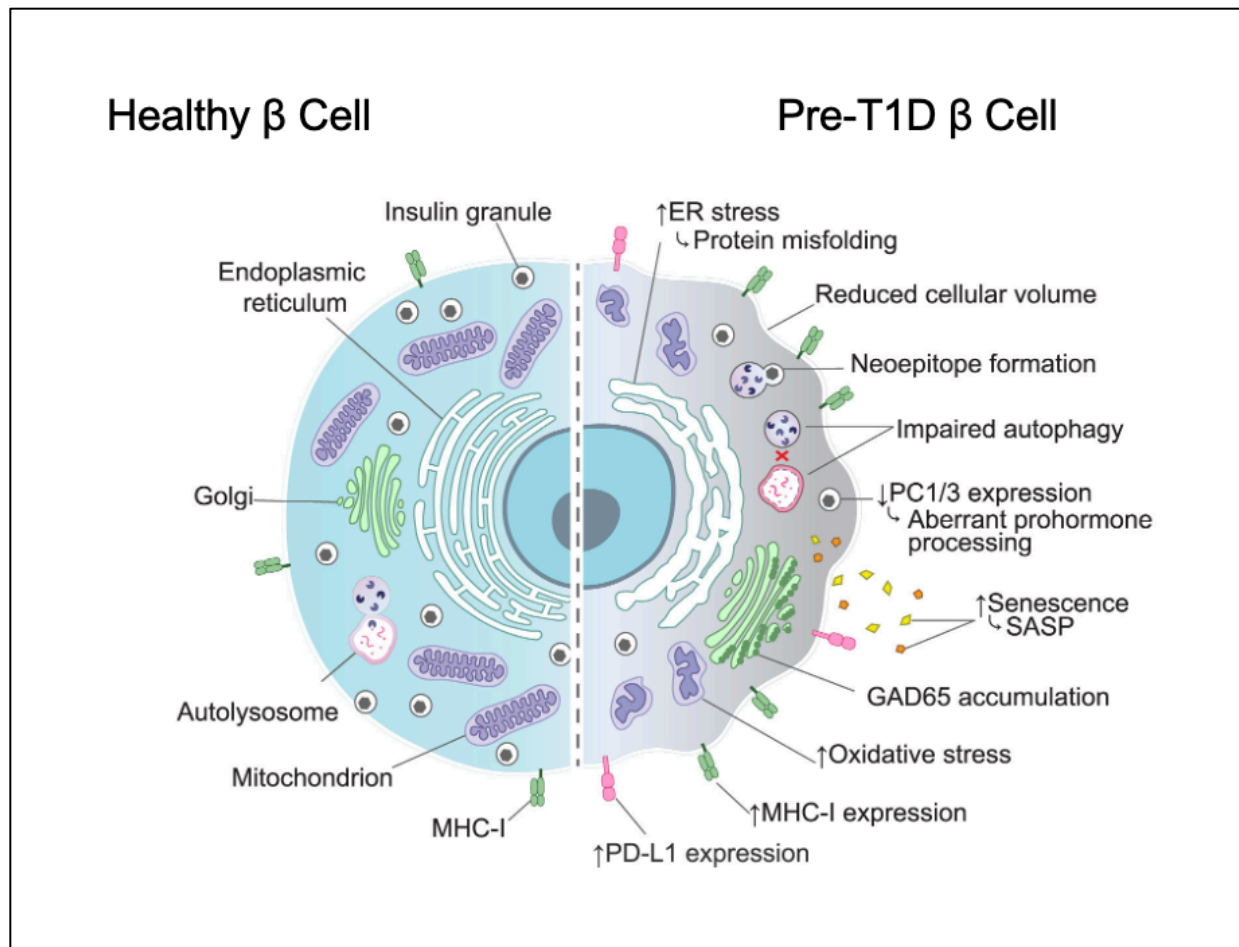


Figure 4: β cell dysfunction precedes T1D.

SOURCE: Reproduced from Atkinson & Mirmira, *Cell Metabolism* (2023)¹⁰⁵ with permission.

ER Stress

The primary function of the β cell is synthesizing and secreting insulin. The insulin gene encodes the 110 amino acid preproinsulin peptide, which is first cleaved at the ER to become proinsulin, and then by several prohormone convertases in the Golgi and secretory granules to become insulin and C-peptide¹⁰⁶. Keeping up with insulin demand is a multi-step, energetically costly process; ATP is required for much of the workflow, from translocation into the ER to chaperone-aided folding and vesicular transport. High metabolic demand (e.g. puberty, growth, insulin resistance) can strain β cell mitochondria, ER, and secretory machinery, leading to breakdowns in insulin processing. Accumulation of misfolded proteins in the ER activates the unfolded protein response (UPR). The UPR has an ‘adaptive’ arm that attempts to restore proteostasis in the cell by increasing levels of protein-folding chaperones (BiP, GRP94), decreasing overall mRNA translation and protein folding burden (phosphorylation of eIF2 α), and activating ER-associated degradation (ERAD) and autophagy to turn over accumulated proteins. If ER stress is prolonged or resolution fails, the UPR can also initiate apoptosis via factors like CHOP and caspases^{107,108}.

Elevated markers of β cell ER stress (BiP, CHOP) have been observed in the pancreata of people with T1D¹⁰⁹ as well as prediabetic NOD mice¹¹⁰, even in islets without immune infiltrates. ER stress could contribute to the development of autoimmunity through several mechanisms. For one, stress upon protein processing machinery makes abnormal posttranslational modifications more likely; these mistakes can lead to the formation of novel peptides that the immune system does not recognize as self, termed ‘neoantigens’^{111–113}. Indeed, so-called hybrid insulin peptides (HIPs) formed from the fusion of proinsulin and other β cell proteins have been shown to stimulate CD4 T cells isolated from the pancreata of individuals

with T1D¹¹⁴. The UPR can also activate transcription of proinflammatory genes downstream of NF- κ B and stimulate type 1 interferon (IFN) production. These inflammatory cascades cause β cells to express additional MHC I and become more ‘visible’ to cytotoxic CD8⁺ T cells, while also releasing chemokines that recruit more immune infiltration to the islet^{115,116}. β cell death induced by prolonged ER stress can also expose damage associated molecular patterns (DAMPs) and β cell antigens, further promoting immune infiltration and activation¹¹⁷.

Oxidative Stress

Another side effect of the heavy biosynthetic demand on β cells is an accumulation of reactive oxygen species (ROS), which are a byproduct of both insulin disulfide bond formation at the ER and the ATP production in the mitochondria required for insulin synthesis. β cells are uniquely ill-equipped to remedy oxidative stress, as they express low levels of antioxidant molecules like glutathione peroxidase, catalase, and superoxide dismutase compared to other secretory cells and even other islet cell types¹¹⁸. ROS accumulation can lead to DNA damage, oxidative modification of proteins (i.e. neoantigen formation¹¹⁹), mitochondrial damage, and triggering of apoptosis or necrosis. Elevated oxidative stress markers have been observed before the development of islet autoantibodies in the serum of humans who went on to develop T1D¹²⁰ as well as in the islets of prediabetic NOD mice¹²¹, all suggesting that oxidative stress may be an early mediator of islet autoimmunity.

Inflammatory Stress & Insulitis

The most common suggestion of a T1D trigger in the literature is viral infection, and prospective cohort studies have provided some evidence of association between enteroviruses and islet autoimmunity^{71,122}. The presence of viral RNA- or other cellular stressors like prolonged ER or oxidative stress- can lead to activation of pattern recognition receptors (PRRs)

in the β cell. One of those PRRs is MDA5, encoded by T1D risk gene *IFIH1*, which sets off an inflammatory cascade culminating in type 1 interferon production, release of immune cell attracting chemokines like CXCL10, and upregulation of antigen processing and presentation machinery. Interferon α (IFN- α) in particular has been dubbed a ‘key trigger’ of T1D pathogenesis^{123–125}. A type 1 interferon response signature is detectable in the blood of those at risk for T1D before islet autoantibody development. Decades of evidence affirm the presence of IFN- α in the islets of those with T1D and demonstrate that IFN- α upregulates β cell expression of HLA-I^{127–129}. IFN- α is produced in part by stressed β cells themselves and more prolifically from plasmacytoid dendritic cells (pDCs). Other innate immune cells like macrophages and conventional DCs are early recruits to the stressed islet and can release additional proinflammatory cytokines like IL-1 β , TNF- α , and IL-6. Natural killer (NK) cells secrete interferon gamma (IFN- γ) and may be able to kill β cells directly upon recognition of stress signals¹³⁰. Finally, in addition to coactivating CD8+ T cell cytotoxicity, activated CD4+ T cells release IFN- γ , IL-1 β , and TNF- α in the islet. Proinflammatory signaling by and acting on the β cell creates a positive feedback loop of ER stress, oxidative stress, β cell death, and continued immune activation.

1.4 Biological sex and the islet

1.4.1 Biological sex and autoimmunity

It is estimated that nearly 1 in 10 people in the United States are living with an autoimmune disease and that a striking ~80% of those people are female (46, XX)^{131,132}. The sex distribution varies by individual autoimmune disease. For example, Sjögrens and systemic lupus erythematosus have a 9:1 female:male prevalence, while multiple sclerosis and rheumatoid arthritis are diagnosed at 3:1. It is thought that the female predominance of autoimmunity results

from a combination of genetic, epigenetic, and hormonal factors. A number of key immune regulatory genes, such as chemokine receptor CXCR3, Treg transcription factor FOXP3, and PRRs TLR7 and TLR8, are expressed on the X chromosome. In females, one of the two X chromosomes in each cell is randomly silenced by the long noncoding RNA XIST, but 15-20% of genes ‘escape’ the inactivation process^{133,134}. One theory posits that a double dose of immune regulation genes might equip females with a more excitable immune system^{135,136}; this notion is supported in part by females’ more favorable outcomes in the settings of vaccination, infections, and cancer^{137,138}. A recent study showed that XIST and its various binding partners form highly immunogenic ribonucleoprotein complexes that can induce autoimmune pathologies even when expressed as a transgene in male mice¹³⁹, reinforcing a role for X chromosome regulation in autoimmunity.

Other hypotheses explore the role of sex hormones in autoimmunity. Female-biased autoimmune diseases tend to occur in the years after sexual maturity, and symptoms are often exacerbated or remitted during periods of hormonal flux such as pregnancy and menopause¹⁴⁰. Effects of sex hormones on the immune system vary by cell type, dosage, and disease context. Estradiol, the most abundant and potent estrogen in females during reproductive years, can stimulate proinflammatory immune function at low doses (e.g. IFN- α production and antigen presentation by pDCs, class switching and autoantibody production in B cells), while also promoting more tolerogenic phenotypes at high doses (e.g. suppression of IFN- γ and cytotoxic function in NK cells, anti-inflammatory M2 macrophage phenotype, decreased CD8+ T cell cytotoxicity)¹⁴¹. Comparison of gene expression in the peripheral blood by sex shows female enrichment of inflammatory pathways like type 1 interferon response compared to males, and the sex difference is exaggerated in women on hormonal birth control¹⁴². Testosterone, the major

male sex hormone, is most often associated with anti-inflammatory immune phenotypes and protection from autoimmune processes (e.g. anti-inflammatory Th2 CD4⁺ T cells, M2 macrophages, and Tregs)¹⁴³.

About half of T1D cases occur in children, and these are diagnosed at approximately equal rates by sex. Contrary to other common autoimmune diseases, however, T1D incidence becomes more common in males after puberty; a metanalysis across 35 countries showed an average male:female incidence ratio of about 1.5:1 after age 15^{144–147}. Thus, traditional ideas about how sex interacts with the immune system (i.e. proinflammatory effects of estradiol or X chromosomes, or protective immunosuppressive effects of testosterone) do not fit with a disease like T1D. One explanation may instead involve the interaction of sex with the *target cell* of T1D, the pancreatic β cell.

1.4.2 Estrogen receptors in the islet

Androgens and estrogens are major hormonal drivers of sexual development and function. Both classes of sex steroids are derived from cholesterol utilizing many of the same enzymes and intermediates, and both are primarily synthesized at the gonads. In response to luteinizing hormone (LH) from the hypothalamus, the theca cells of the ovary convert cholesterol to the androgens androstenedione and testosterone, which can be converted to estrogens estrone (E1) and estradiol (E2) in the granulosa cells by the aromatase enzyme. In males, the Leydig cells of the testes convert cholesterol to testosterone. Sex steroids can also be synthesized and modified in non-gonadal tissues. It is well known that the adrenal cortex synthesizes androgens, adipose tissue and bone express the aromatase enzyme, and target tissues can modify testosterone to the more potent dihydrotestosterone using 5 α -reductase. β cells have been shown to express sex steroid receptors and the machinery of sex steroid synthesis, suggesting that they can produce and interact with these molecules locally¹⁴⁸.

There are three known estrogen receptor subtypes, all of which have been detected in β cells¹⁴⁸. ER α and ER β are nuclear receptors which homodimerize upon binding to their ligands, binding to the DNA at estrogen response elements (EREs) and activating transcription. Interestingly, some these receptors can also be associated with the plasma membrane via palmitoylation or caveolins, representing a pool available for rapid nongenomic signaling via kinase cascades or other second messengers¹⁴⁹. ER α activity in the β cell is associated with higher insulin content and secretion¹⁵⁰. Also membrane-localized is the third type of estrogen receptor, g-protein coupled estrogen receptor 1 (GPER1), which signals non-genomically through cyclic AMP, calcium mobilization, and protein kinase A to promote insulin secretion and cell survival in the β cell^{151,152}.

1.4.3 Estradiol protects against β cell stress

Several lines of evidence suggest that female sex is protective of β cell health and function. Clinically, females show lower incidence T1D and T2D, both of which involve failure and death of the β cell^{145,147}. Even when diagnosed with T1D, females exhibit higher residual C-peptide levels at diagnosis, indicative of higher residual insulin production^{153,154}. Single cell RNA sequencing of human islets also reveals that compared to female islets, male islets from nondiabetic donors already show amplified signature of ER and oxidative stress¹⁵⁵. Whether this relative female protection is mediated by estradiol or other sex characteristics is not entirely clear. It has long been observed that E2 has insulinotropic effects on the pancreatic β cell¹⁵⁶, and mounting evidence demonstrates that E2 protects the integrity of β cell health and function in the face of numerous stressors.

Female mice are resistant to several diabetes-modeling insults. They maintain higher glucose tolerance on high-fat diets¹⁵⁷ and maintain normal β cell mass after treatment with streptozotocin (STZ), which induces diabetes in male mice via DNA damage, oxidative stress,

and β cell death¹⁵⁸. Underscoring the importance of estradiol in this protection, knockout of ER α or of the aromatase enzyme, which converts androgens to estrogens, susceptibilizes female mice to STZ-induced diabetes¹⁵⁸. Estradiol's protective role is further supported by *in vitro* studies wherein pretreatment with estradiol renders islets more resilient to glucolipotoxic, oxidative, and inflammatory stresses^{158–160}. *In vivo* studies are in agreement with these findings; estradiol treatment of isolated human islets improved their subsequent engraftment, survival, and restoration of euglycemia in insulin-deficient mice¹⁶¹, and exogenous estradiol treatment prevented or delayed autoimmune diabetes in NOD mice¹⁶².

Estradiol is known to potentiate anti-apoptotic signaling (Bcl-2), reduce ROS accumulation (SOD, catalase), protect mitochondrial function, and suppress inflammatory signaling (NF- κ B)^{160,163,164}. Which of these mechanisms might be at play in female protection from islet autoimmunity and T1D- and through actions in which cell type(s)- are not known.

1.5 Summary and Hypothesis

In summary, T1D is an insulin-deficient form of diabetes mellitus driven by the autoimmune destruction of pancreatic β cells. While T1D can be well-managed with exogenous insulin and glucose monitoring, it remains a life-altering disease with serious complications. Genome-wide association studies and families with T1D have elucidated genetic (i.e., HLA haplotypes, mutations in *INS* or inflammatory genes) and environmental (i.e., viral infection) risk factors for T1D, yet exactly how these interact to result in loss of immune tolerance to β cells is still under investigation. Increasing evidence suggests that β cell stress precedes and contributes to the development of islet autoimmunity. T1D is unusual among autoimmune diseases in that incidence is more common in *males*, particularly after the age of puberty. Numerous lines of clinical and experimental evidence suggest that female sex- particularly the sex hormone estradiol- protects β cells from stress-induced death.

In this dissertation, I investigate the hypothesis that chronic exposure to estradiol changes how islets respond to inflammatory stress and how they interact with immune cells, preconditioning them to remain less immunogenic. I utilize human islets from male and female donors treated with combinations of proinflammatory cytokines and estradiol, as well as exogenous estradiol treatment in the NOD model of T1D to explore islet intrinsic and cell-type specific responses to estradiol and inflammation. With this work, I aim to illuminate islet intrinsic features that might protect against disease progression and complement immunosuppressive approaches in individuals at risk for T1D.

CHAPTER 2: RESEARCH DESIGN AND METHODS

2.1 Human islet culture

De-identified non-diabetic human islets from cadaveric donors were provided by the Integrated Islet Distribution Program (IIDP) and the University of Alberta Diabetes Institute Islet Core (Edmonton, Alberta, Canada) (**Table 2**). Culture medium used for human islet experiments was Prodo Islet Media (Standard) supplemented with human AB serum, glutamine and glutathione (Prodo), and ciprofloxacin (Fisher). Islets were cultured in the presence or absence of a proinflammatory cytokine (PIC) cocktail containing 1000 IU/mL human IFN- γ (R&D Systems; 285-IF-100) and 50 IU/mL human IL-1 β (R&D Systems; 201-LB-005), or with 10nM 17- β Estradiol (Millipore Sigma, E2758) for 18 hours.

2.2 Bulk RNA sequencing

Human islet RNA stored in RLT plus buffer containing β -mercaptoethanol was extracted using RNAeasy Plus Micro kit (Qiagen) according to manufacturer instructions. Samples were submitted to the University of Chicago Genomics Core for sequencing using the NovaSeq-X system (Illumina). FASTQ files generated by the core were then analyzed by ROSALIND®, with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA). Reads were trimmed using cutadapt¹⁶⁵. Quality scores were assessed using FastQC. Reads were aligned to the Homo sapiens genome build hg19 using STAR¹⁶⁶. The raw counts matrix was run through ComBat-Seq¹⁶⁷ for batch correction utilizing default parameters to account for high variability between human samples. DEseq2 was used to calculate fold changes and p-values and perform optional covariate correction¹⁶⁸. Overrepresentation analyses were performed using Enrichr^{169–171}. Gene set enrichment analysis was performed using the GSEA 4.3.2 software¹⁷². Rosalind was

also used to analyze bulk sequencing from previously published studies^{173–176}, two of which are available via the NCBI's Gene Expression Omnibus ([GSE169221](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169221), [GSE226888](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226888)).

2.3 GSEA of HPAP data

FASTQ files of 10x Genomics scRNA-Seq data for human islets from nondiabetic, AAB+, and T1D donors were downloaded from the data portal of the HPAP (<https://hpap.pmacs.upenn.edu>)¹⁷⁷. Cell Ranger version 6.1.2 was used to perform quality control and align reads to human genome build GRCh38¹⁷⁸. SoupX version 1.6.1¹⁷⁹ was used to remove ambient mRNA, and doublets were removed with scDblFinder version 3.16¹⁸⁰. Cells retained for further analysis were those with 200-9,000 detected features, <25% mitochondrial reads, and fewer than 10,000 counts. Seurat function SCTransform^{181,182} was used to normalize counts, removing effects of library depth and regressing out the variation from the mitochondrial reads' ratio. Principal component analysis was performed using the top 3,000 variable genes, and Harmony 0.1.1¹⁸³ was used to integrate all samples based on the top 50 principal components, setting donor identity and reagent kits as primary confounders. Human islet cell types were annotated using scScorter version 0.0.2¹⁸⁴. A total of 10,167 β cells remained after all above steps, including 4,730 cells from nondiabetic donors, 2,372 cells from single-autoantibody-positive donors, 2,480 cells from double-autoantibody-positive donors, and 585 cells from type 1 diabetic donors. GSEA was performed in R using the msigbdr package (<https://igordot.github.io/msigbdr/>) using the Hallmarks Gene Sets.

Murine cell and islet culture and treatment

To isolate mouse islets¹⁸⁵, collagenase was injected into the pancreatic bile duct to inflate the pancreas, which was then removed and dissociated via rocking and syringe mixing in a 50 mL tube. Dissociated tissues were then placed within a gradient of Histopaque and HBSS and

centrifuged at 2080 rpm for 18 minutes. After passing the supernatant through a 70 μ M filter, the islets were rinsed into a culture plate in RPMI medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Healthy islets were handpicked under microscopy and transferred to fresh media, after which they were allowed to recover at 37°C and 5% CO₂ overnight before experimentation.

Murine β cell line MIN6¹⁸⁶ was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 15% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. MIN6 cells and mouse islets were treated with a proinflammatory cytokine cocktail containing 25 ng/mL mouse IL-1 β (R&D Systems; 401-ML-010), 50 ng/mL mouse TNF- α (R&D Systems; 410-MT-010), and 100 ng/mL mouse IFN- γ (R&D Systems; 485-MI-100) for 18 hours, or with 17- β Estradiol (Millipore Sigma, E2758) (concentration and duration detailed in each experiment).

2.5 Quantitative real-time PCR

RNA was extracted from samples stored in Qiagen RLT buffer containing β -mercaptoethanol using the RNeasy Mini Kit. For cDNA synthesis, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Taqman primer-probe mixes were used to detect desired mRNA targets via quantitative PCR using Bio-Rad CFX Opus. Relative gene expression was calculated using the comparative threshold cycle value (CT). Data are displayed normalized to expression of *Actb* and then to the average of control group expression (ddCT).

2.6 Mass spectrometry-based proteomics

Human pancreatic islet samples were lysed in Qiagen RLT Buffer + 1% beta mercaptoethanol, homogenized with several passages through a 25 $\frac{1}{2}$ gauge needle and

QiaShredder column, and run over a genomic DNA depletion column. 1 volume of 70% ethanol was added to each flow through, and this was run over the RNeasy Micro column after mixing. The flow-through from this step was then subjected to the following to precipitate protein: Added 4 volumes of ice-cold acetone to the flow-through from the RNeasy MinElute spin column. Incubated for 30 min at -20°C . Centrifuged for 10 min at full speed in a benchtop centrifuge. Discarded the supernatant and air-dried the pellet. Stored at -80°C until shipping to Pacific Northwest National Laboratories on dry ice.

A data-independent acquisition approach was used for the quantitative proteomics analysis¹⁷³. Human islet lysates were suspended in 8 M Urea; 50 mM Tris pH 8.0; 75 mM NaCl; complete protease inhibitor cocktail (Sigma 5892791001) buffer. Disulfide bonds were reduced by adding dithiothreitol (5 mM final concentration), followed by alkylation step using iodoacetamide (final concentration of 40 mM). Samples were diluted 4-fold with 50 mM Tris HCl (pH 8.0) and digested with an 1/50 (enzyme/protein) ratio of sequencing grade endoproteinase LysC (VWR) for 2 h at 25°C , followed by sequencing grade trypsin (Promega) overnight at 25°C . Final concentration of 1% trifluoroacetic acid was used to quench the reaction, followed by desalting with C18 using 15-mg C18-E Strata 96-well plate, and peptide concentrations were estimated with a BCA assay. Samples were diluted to 0.1 $\mu\text{g}/\mu\text{l}$ for LC-MS/MS DIA. Individual samples were analyzed by data-independent acquisition using liquid chromatography with tandem mass spectrometry (LC-MS/MS) on QExactive HF mass spectrometer (Thermo Fisher Scientific). Peptides were separated in a C18 column (70 cm 3 75 mm i.d., Phenomenex Jupiter, 3 mm particle size, 300 Å pore size) connected to an Acquity M-Class Nano UHPLC system (Waters). Tandem mass spectra were collected from the range of 400–900 m/z with 10 m/z increment windows (10.0 m/z isolation width; 30% normalized collision energy; 70,000 resolution at 400 m/z). Post-LC-

MS/ MS, a Insilco library was created using the human Swissprot database (September 12, 2024, 20,656 sequences) on our inhouse DMS data analysis interface using DiaNN. Only fully tryptic peptides were considered with up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification; acetylation of protein N-terminus and oxidation of methionine residues were set as variable modifications. Precursor peptide length was set to a range of 7-30, m/z 400-900 and charge 2-4. The final library contained peptides filtered with 1% false discovery rate (FDR) at protein, peptide, and peptide/spectrum match levels. The DIA sample runs were processed in the same workflow matching against the generated Insilco library, using an FDR of 1%.

Data was evaluated for potential sample outliers using a robust Mahalanobis distance (RMD) measure based on correlation with samples from the same treatment group, median absolute deviation (MAD), proportion of observations missing, and skewness of sample profiles¹⁸⁷. Sparse proteins without enough data to conduct a statistical test were filtered from the dataset. A protein was required to have a non-missing value for the control treatment and at least one other treatment, within the same donor, for at least two donors. Data was normalized via median centering. Comparisons of mean protein and gene expression of each group back to the control group were performed. Datasets were analyzed with all samples and also broken into separate datasets for males and females only and analyzed for these subsets. A generalized linear model with treatment group as a fixed effect and donor as a random effect was fit to each protein using the *lme4* package in R¹⁸⁸. The mean for each treatment group was compared to the control group for differences in mean abundance, and a Holm multiple comparison adjustment was conducted¹⁸⁹. Enrichr was used to perform overrepresentation analyses^{169–171}.

2.7 Animals and procedures

All mice were housed in a specific-pathogen-free facility at the University of Chicago with ad libitum access to food and water. NOD/ShiLtJ (NOD) (#001976) and C57BL/6J (#000664) mice were purchased from The Jackson Laboratory.

6-week-old male and female NOD mice were implanted subcutaneously with a 90-day, 0.5 mg pellet containing 17- β Estradiol or matched placebo. For diabetes incidence experiments, blood glucose was monitored up to twice weekly using an AlphaTrak glucometer beginning at 10 weeks of age. Mice were euthanized after diabetes incidence (two consecutive readings > 250 mg/dL) or at 25 weeks of age, whichever came first. For insulinitis, β cell mass, and scRNAseq studies, mice were euthanized at 10 weeks of age. Pancreata were collected for islet isolation or for histology.

2.8 Estradiol ELISA

Serum was first mixed 4 parts methanol: 1 part serum and incubated at room temperature for 10 minutes before being centrifuged at 2,000 x g for 10 minutes. Supernatants were then transferred to a clean tube and dried under inert gas. 17- β estradiol was then measured using an Estradiol ELISA kit (Cayman, #501890) using manufacturer instructions.

2.9 Immunohistochemistry and quantification

Pancreata were fixed in 4% paraformaldehyde and embedded in paraffin, then serial 5 μ m sections were cut from 3 levels (100 μ m apart). 3 sections per mouse (1 from each level) were stained with anti-Insulin primary antibody (ProteinTech, 15848-1-AP at 1:200 dilution). Label was developed using ImmPRESS peroxidase-conjugated secondary antibody and DAB peroxidase substrate (Vector Laboratories), followed by hematoxylin counterstaining. Slides were imaged using the Keyence BZ-X810 fluorescence microscope system. BZ-X800 Analyzer was used to quantify insulin positive area and total pancreas area. β cell mass was calculated by

multiplying (insulin positive area/total pancreas area) by pancreas weight measured at the time of euthanasia.

Insulinitis scoring was performed by assessing the extent of immune cell infiltration at each islet: 1 = no immune infiltrates, 2 = infiltrates cover <50% of area, 3 = >50% area covered by infiltrates, 4 = infiltration over 100% of islet.

2.10 Single cell RNA sequencing

NOD mouse islets were isolated as described above, then dissociated into single cells by incubating in accutase solution containing DNase and RNasin (Promega) for 8 minutes at 37°C. Suspension was then transferred to a 5mL polystyrene tube containing 1% bovine serum albumin HBSS and spun at 300 x g for 5 minutes at 4°C, washed once more BSA-HBSS, and spun again.

Single cell suspension was submitted to the University of Chicago Genomics Facility for 10x library generation using Chromium Single Cell 3' v 3.1, followed by sequencing on Illumina NovaSeq-X. Cell Ranger was used to align reads to the *Mus musculus* genome build GRCm39, count unique molecular identifiers (UMIs), call cell barcodes, and perform default clustering. Ambient mRNA was removed using SoupX, version 1.6.1, and clustering was performed utilizing Seurat version 5.1.0 in R version 4.3.1. Cells were filtered to those with at least 500 unique features and <5% mitochondrial genes. Replicates from each treatment group (Estrogen vs. Placebo, n=3 each) were merged using SCTransform, and integration was performed using Harmony version 1.2.3. Clustering was performed using Seurat and visualized by UMAP, and cluster markers were identified using FindAllMarkers (Wilcoxon's rank-sum test) in Seurat. Differential gene expression was analyzed using FindMarkers, and GSEA was performed using the ClusterProfiler package, version 4.10.1. Several data visualizations were created using ggplot2 version 3.5.1.

2.11 Statistics

For comparisons involving 2 conditions, a 2-tailed student's unpaired t-test was performed. A Mantel-Cox log-rank test was performed to compare diabetes incidence between treatment groups. GraphPad Prism, version 10, was used for these statistical analyses and corresponding visualizations. Statistical significance was defined as $P < 0.05$. Statistics used for analyses of RNAseq and proteomic analyses are described in more detail above.

CHAPTER 3: HUMAN ISLETS RESPOND DIFFERENTLY TO PROINFLAMMATORY CYTOKINES ON THE BASIS OF DONOR SEX

3.1 Introduction

Considerable progress has been made in identifying genetic and environmental features shared by individuals who develop T1D, perhaps most notably the high-risk HLA haplotypes (DR3-DQ2, DR4-DQ8)⁵⁸. On the other hand, the vast majority of individuals with these haplotypes do not go on to develop T1D^{64,65}. The specific ‘triggers’ of disease onset in those at risk for T1D remain a fundamental gap in our understanding of T1D pathogenesis.

A widely held theory posits that viral infection- and the resulting inflammation and contact between β cells and the immune system- may set off a cascade toward islet autoimmunity^{190,191}. There is some evidence for an association between viral infection and progression to T1D^{70,122}, and more extensive evidence for the broader association between inflammation and T1D onset^{126,192,193}. T1D involves several inflammatory features derived from both innate and adaptive immune cells. This is demonstrated by the presence of immune infiltrates in T1D islets, the development of autoantibodies against islet antigens, the dominance of HLA haplotypes in the heritability of T1D, and the proinflammatory cytokine signatures observed in the serum and islets of individuals at risk for T1D even prior to autoantibody development^{126,127,194}. In recent years, a broadening contingent of T1D researchers is revisiting a possibility raised decades earlier; namely, that the β cell, owing to its dysfunction or the manner in which it responds to an environmental insult like inflammation, may contribute to its own immune-mediated death^{100–104}. More than half of T1D candidate genes are expressed in the β cell¹⁹⁵, and genes and proteins upregulated in β cells themselves downstream of proinflammatory cytokine signaling, have been shown to render them more ‘immunogenic’^{196–198}.

T1D, unlike many other autoimmune diseases, is more common in men, particularly after the age of puberty^{144–146}, and the sex hormone estradiol has been shown to protect β cells from apoptosis induced by inflammatory stress¹⁶⁰. In this chapter, I explore the hypothesis that sex influences how the β cell responds to acute inflammatory stress, and thus whether the β cell becomes more immunogenic. To this end, I utilize bulk RNA sequencing and proteomics of human islets challenged with proinflammatory cytokines IL-1 β and IFN- γ - important components of the T1D milieu^{88,195}- to examine whether their response differs by donor sex.

3.2 Results

3.2.1 Human islets from male donors show a broader inflammatory response to IL-1 β and IFN- γ than female islets in a publicly available dataset.

In the setting of T1D, the presence of proinflammatory cytokines both in the circulation and in the islet microenvironment is well-established^{126–128,199}. The sources of these cytokines are likely both innate and adaptive immune cells- IFN- α , TNF- α , and IL-1 β from dendritic cells and macrophages arriving early in the stressed islet, and later IFN- γ from NK cells, CD4+ T cells, and CD8+ T cells²⁰⁰. Treatment of human islets with a combination of IL-1 β and IFN- γ *in vitro* has been shown to upregulate transcriptomic pathways for inflammatory signaling, antigen presentation, and cell death, including T1D candidate genes such as *IFIH1*, *PTPN2*, and several HLA genes¹⁹⁵. This cytokine combination also induces immune cell-attracting chemokine secretion¹⁹⁵ as well as over 100 alternative RNA splicing events likely to alter protein structure and thus produce neoantigens¹⁷⁴.

To interrogate whether the qualitative or quantitative scope of the islet response to proinflammatory cytokines might be shaped by sex, I first turned to a publicly available bulk RNA sequencing dataset performed on human islets treated with IL-1 β and IFN- γ ¹⁷⁴. These

human islets were collected from 10 non-diabetic cadaveric donors (n=6 males, 4 females); a subset of islets from each donor was left untreated as a control (Veh) and a subset was treated with proinflammatory cytokines (PIC) for 24 hours prior to RNA isolation and sequencing (**Figure 5A-B**). Notably, this dataset was not created with comparison by sex in mind, and the donor characteristics were not well-matched by sex (**Table 1**). I thus approached this dataset as a ‘discovery’ set to generate hypotheses to be tested in a well-matched validation set.

In its original publication, this data was used to study the impact of PIC on alternative RNA splicing events in the islet¹⁷⁴. Including islets from all 10 donors and filtering for genes with a fold-change ≥ 1.5 or ≤ -1.5 and an adjusted p-value < 0.05 , I identify 3793 differentially expressed genes (DEGs) in response to PIC (**Figure 5C**). By overrepresentation analysis, these DEGs map to expected inflammatory pathways, including IFN- γ and IFN- α responses and Allograft Rejection among many others (**Figure 5D**). Intriguingly, separating the analysis by sex reveals that 3754 genes are differentially expressed in PIC-treated male islets, while only 1117 are differentially expressed in PIC-treated female islets compared to vehicle. Juxtaposing these responses, 1016 DEGs were shared between sexes, a striking 2738 were unique to male islets, and only a meager 101 were unique to female islets (**Figure 6A-C**). Functional pathways represented among the 2738 male-specific DEGs related to inflammation and cell death (TNF- α Signaling via NF- κ B, IL-6/JAK/STAT3 Signaling, Apoptosis), and some also related to cell identity and development (Notch Signaling, Wnt- β catenin Signaling, Pancreas Beta Cells, Epithelial Mesenchymal Transition). Among the 101 female-specific DEGs were a small number of genes related to Inflammatory Response (**Figure 6D**). The large number of male-specific, proinflammatory DEGs may represent a broader, more aggressive response in male islets to an identical inflammatory challenge.

Donor ID	Islet Source	Age	Sex	BMI	HbA1c (%)	Islet Purity (%)
SAMN08769390	IIDP	33	Female	34.2	5.5	95
SAMN08769199	IIDP	43	Female	29.5	5.7	80
SAMN08769206	IIDP	52	Female	25.9	5.6	90
SAMN08769129	IIDP	44	Female	34.5	Unknown	90
SAMN08769191	IIDP	35	Male	24.2	5.4	95
SAMN08769188	IIDP	30	Male	35.9	5.4	90
SAMN08769132	IIDP	23	Male	24.8	5.3	95
SAMN08769121	IIDP	28	Male	24.6	5.1	95
SAMN08769124	IIDP	15	Male	24.6	5.1	80
SAMN08769073	IIDP	28	Male	30.7	4.9	95

Table 1: Islet donor characteristics for discovery dataset.

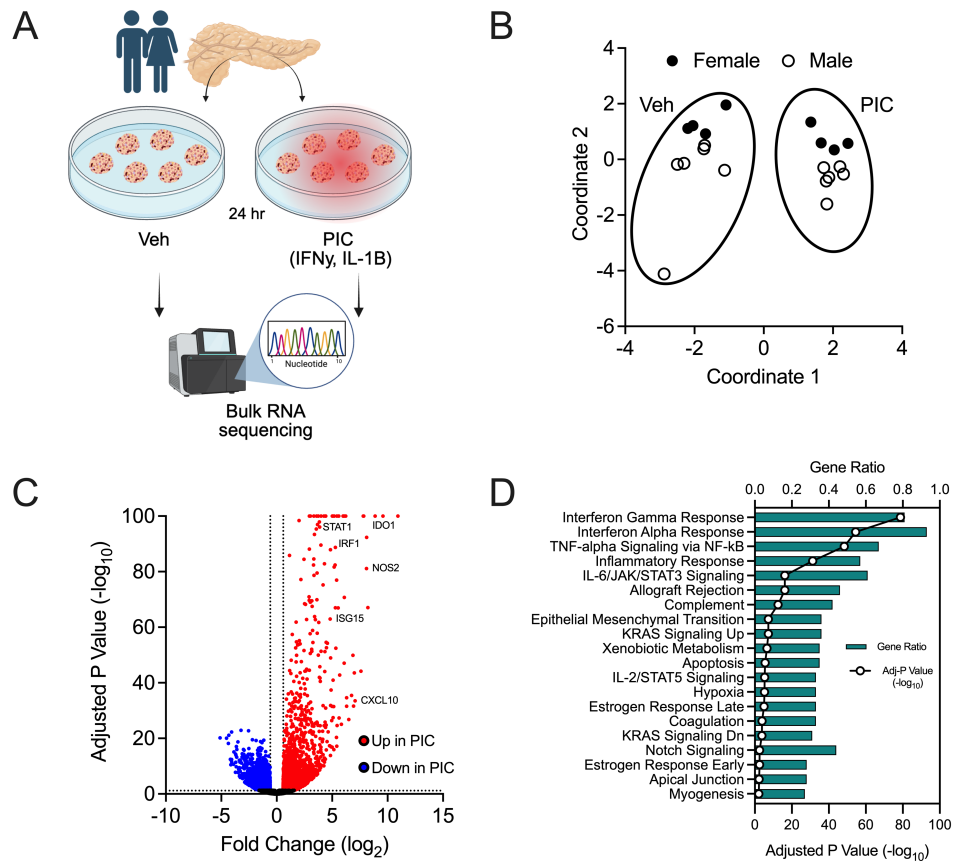


Figure 5: Human islets treated with PIC upregulate canonical inflammatory gene pathways.

(A) Schematic of human islet treatments (nondiabetic donors, n=6 male, 4 female) performed by Wu et al¹⁷⁴. Human islets from each donor were treated with vehicle (Veh) or with IL-1 β and IFN- γ (PIC) for 24 hours prior to RNA isolation and bulk RNA sequencing. (B) Multi-dimensional scaling (MDS) plot of all samples included in the analysis. Created with BioRender.com. (C) Volcano plot of all differentially expressed genes (PIC vs. Veh). Colored circles represent DEGs with fold-change ≥ 1.5 or ≤ -1.5 and an adj-p-value < 0.05 . (D) MSigDB Hallmarks pathways modulated by PIC treatment identified via overrepresentation analysis. Adj-p-value < 0.05 for all pathways.

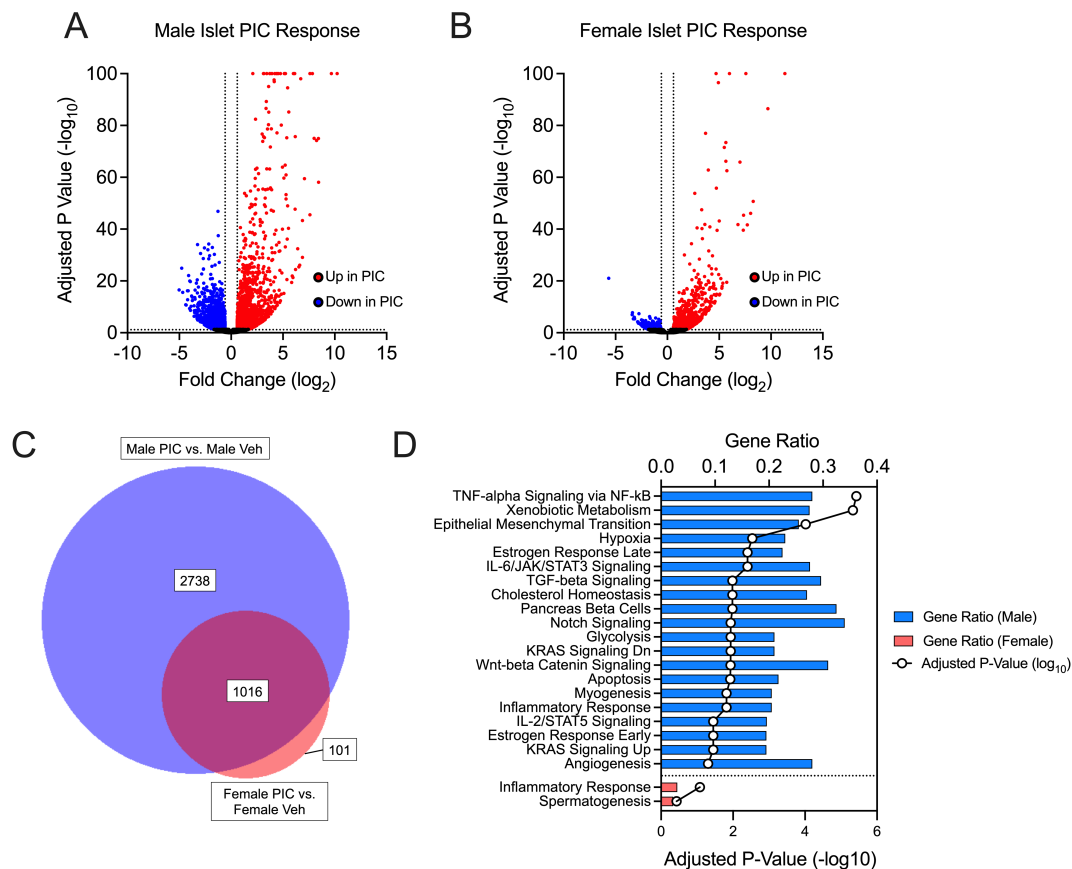


Figure 6: Islets from male donors show a larger inflammatory response to PIC.

(A) Volcano plot of PIC response in male islets (PIC vs. Veh). Colored circles represent DEGs with fold-change ≥ 1.5 or ≤ -1.5 and adj-p-value < 0.05 . Red = Up in PIC, blue = down in PIC. (B) Volcano plot of all PIC response in female islets (PIC vs Veh). (C) Venn diagram of DEGs in the male and female PIC responses. (D) Overrepresentation analysis of male-specific DEGs (blue bars) and female specific DEGs (pink bars). Adj-p-value < 0.05 for all Male pathways.

3.2.2 GSEA reveals enriched IFN- α response in male islets and enriched EMT in female islets.

When comparing male and female islet transcriptomes directly using the same strict filters (FC >1.5, adj-p-value < 0.05), the small number of DEGs identified were restricted to the sex chromosomes (i.e. TSIX on the X chromosome; TTTY15, ZFY, UTY on the Y chromosome) in both the PIC and vehicle conditions. Gene set enrichment analysis (GSEA) is a computational method that compares the expression of *all* detected genes between two selected groups with no arbitrary cutoff for fold-change or significance. GSEA instead creates a ranked list- in this case, ranked from genes most prevalent in female samples to genes most prevalent in male samples- and asks whether genes in predefined functional categories ('gene sets') tend to fall near the top or bottom of that list. Using GSEA, PIC-treated male islets show an enrichment of IFN- α response genes compared to PIC-treated female islets (**Figure 7**). IFN- α has been called a 'key trigger' for T1D¹²³, and several genes involved in IFN- α response are considered T1D candidate genes. Notably, the leading edge- the genes contributing most strongly to the enrichment of this pathway in male islets- included T1D candidate gene *IFIH1*, as well as genes like *ISG15*, *IL15*, and *CASP8*, which contribute to β cell death in animal models of T1D and whose protein products are elevated in people with T1D^{201–205}.

Female islets, on the other hand, showed an enrichment in genes involved in the epithelial-mesenchymal transition (EMT) pathway compared to male islets (**Figure 7**). Unlike the male bias in IFN- α response, this female bias in EMT gene expression was present in both vehicle and PIC conditions. Through EMT, epithelial cells tend to loosen cell-cell contacts, lose apical-basal polarity, and take on more migratory mesenchymal phenotypes. EMT has been implicated in embryonic development, wound healing and tissue regeneration, and in cancer progression and metastasis²⁰⁶. Whether EMT has a physiologic role in the islet outside of development is unclear, but EMT *has* been observed in β cells undergoing dedifferentiation- a

loss of 'β cell identity' (see Section 1.1.4)- in prolonged cell culture and also in models of diabetes^{207–210}. While dedifferentiation is thought to be a mechanism of β cell failure in T2D^{20,21}, it has been shown to *protect* β cells from autoimmune destruction in the NOD model of T1D by decreasing expression of autoantigens and MHC I^{211,212}. The relative enrichment of EMT genes in female islets may represent shifting endocrine cell identity or other forms of islet remodeling, and whether this might lend protection from autoimmunity requires further investigation.

Overall, re-analysis by sex of this published dataset reveals a sexually dimorphic response of the islet to proinflammatory cytokines, with male islets exhibiting a more aggressive response, including through pathways of known consequence for T1D development (i.e. IFN-α). In comparison to male islets, female islets displayed an EMT signature that may represent β cell dedifferentiation or heightened capacity for islet remodeling. These data suggest that sex preconditions the islet to respond more-or-less adaptively to the same inflammatory insult, which may translate to more or less islet immunogenicity. Of note, this dataset was not created with comparison by sex in mind. As a result, donor characteristics between males and females (i.e. age, BMI, HbA1c) were not matched (**Table 1**). Age and exposure to high glucose are well-known to induce transcriptomic changes in the pancreatic islet in rodent models and in human tissues and are thus potential confounders of this analysis^{213–216}. To address this shortcoming, I next collected islets from a set of well-matched male and female donors to create a validation dataset.

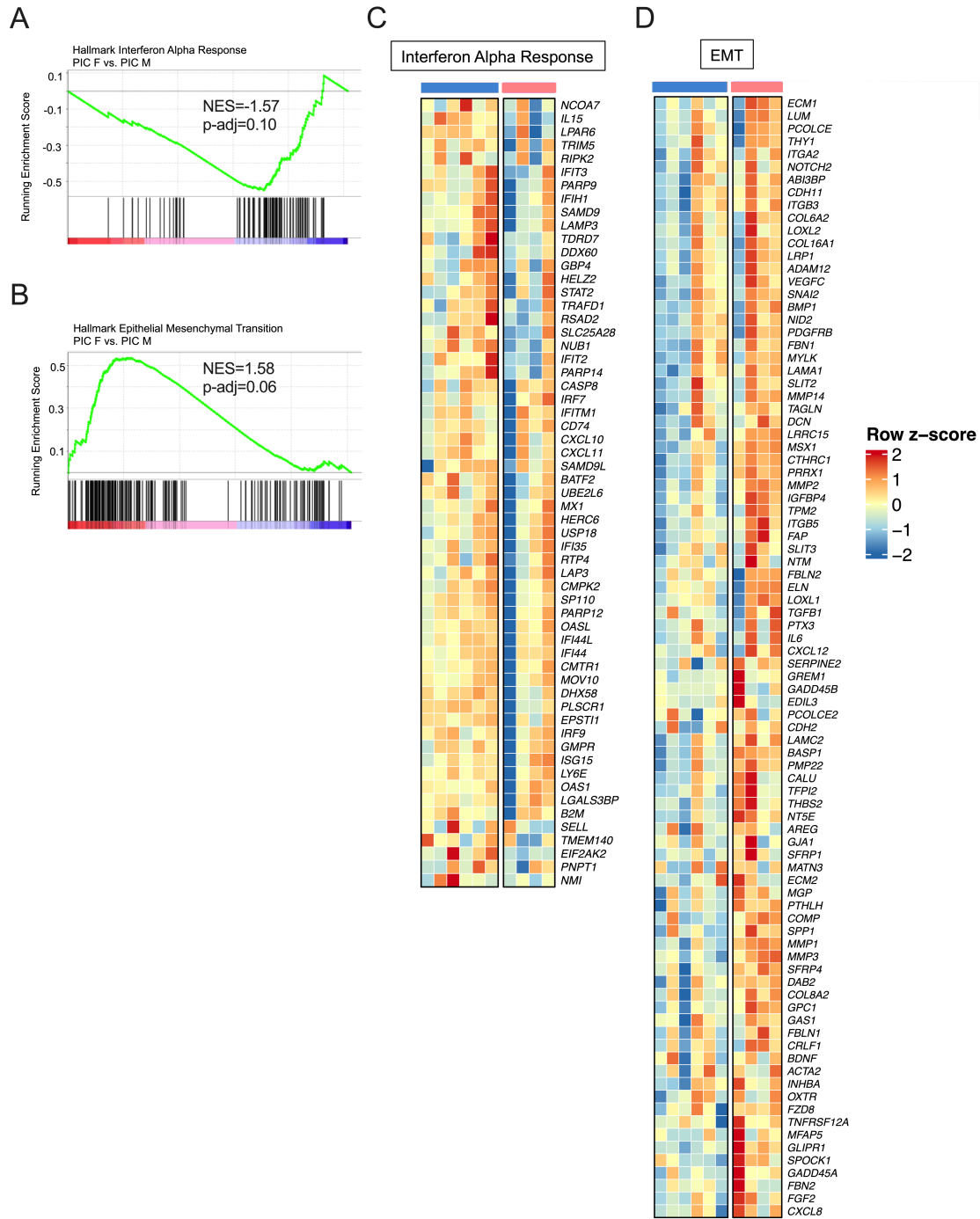


Figure 7: IFN- α genes are enriched in PIC treated male islets, while EMT genes are enriched in vehicle and PIC female islets.

GSEA plots of (A) Hallmarks Interferon Alpha Response pathway and (B) Hallmarks Epithelial Mesenchymal Transition pathway in Male PIC vs Female PIC. (C-D) Heatmaps depicting relative expression of the leading edge genes for (C) Interferon Alpha Response and (D) Epithelial Mesenchymal Transition.

3.2.3 A validation dataset reproduces broader male inflammatory response to PIC.

To validate the findings from 3.2.1, I next performed bulk RNA sequencing on a set of human islets (n=10 males, 10 females) well-matched for age, BMI, and HbA1c levels (**Table 2**). These islets were prepared similarly to the dataset analyzed in Section 3.2.1, with a subset of islets from each donor untreated as control (vehicle) and another treated with IL-1 β and IFN- γ for 18 hours (PIC). Including samples from all 20 donors and using the same gene filters as before (FC >1.5, adj-p-value<0.05), I identify 2134 DEGs in response to PIC. As expected, overrepresentation analysis shows that these DEGs belong to many of the same proinflammatory pathways noted in the prior analysis, including IFN- α and IFN- γ Response, and Allograft Rejection (**Figure 8C**). By sex, the number of DEGs in PIC treated male islets was again larger than that in PIC treated female islets (2386 in males; 1765 in females), and males showed over 3.5 times more sex-specific DEGs than females (864 in males; 243 in females) (**Figure 8D**). Male-specific DEGs related to pro-inflammatory signaling (i.e. TNF- α Signaling via NF κ B; genes including *NFKB1*, *RELB*, *TLR2*, and others) as well as Epithelial Mesenchymal Transition (driven by downregulation of genes like *COL3A1*, *LAMA2*, and *SPARC*) (**Figure 8E**). Coupled with the enrichment in EMT observed in female islets in the discovery set (**Figure 7**), the latter suggests that processes related to EMT may be suppressed in male islets in response to cytokines while they are maintained in female islets. Female-specific DEGs, on the other hand, related primarily to Estrogen Response (e.g. ER α co-regulators *TFAP2A* and *TFAP2C*; ER α target genes *AGR2*, *SERPINA3*) (**Figure 8E**). Overall, these data reinforce the pattern observed in the discovery set of an exacerbated pro-inflammatory response in male islets, while also introducing the possibility that this dimorphism is influenced by sex hormone signaling.

Donor ID	Islet Source	Date Received	Age	Sex	BMI	HbA1c (%)	Islet Purity (%)
SAMN3456133	IIDP	4/27/23	20	Male	5.5	38.5	85
R486	ADI Isletcore	5/9/23	40	Male	5.4	30.4	95
SAMN3584821	IIDP	6/27/23	22	Male	5.1	31.79	90
SAMN3670481 9	IIDP	7/28/23	52	Male	4.9	25.9	85
SAMN3752902 5	IIDP	9/26/23	64	Male	4.8	23	90
SAMN3763859 6	IIDP	10/3/23	34	Male	5.5	27.2	85
R512	ADI Isletcore	10/17/23	63	Male	5.9	34.7	90
R514	ADI Isletcore	10/24/23	57	Male	4.2	26.7	95
SAMN3811742 8	IIDP	11/7/23	47	Male	5.3	28.1	95
SAMN3875092 7	IIDP	12/12/23	34	Male	5.4	24.4	95
R496	ADI Isletcore	6/27/23	68	Female	5.6	22.3	95
R498	ADI Isletcore	7/6/23	49	Female	5.2	28.5	80
SAMN3682322 7	IIDP	8/8/23	41	Female	5.2	38.4	75
R505	ADI Isletcore	8/30/23	53	Female	5.4	33.1	80
R507	ADI Isletcore	9/6/23	59	Female	5.3	19.9	85
R517	ADI Isletcore	11/8/23	41	Female	5.7	27.3	90
SAMN3822666 1	IIDP	11/14/23	37	Female	5.1	30	85
R521	ADI Isletcore	12/5/23	29	Female	5.1	31	70
SAMN3946136 9	IIDP	1/18/24	53	Female	5.6	24.7	90
SAMN4016099 4	IIDP	2/28/24	34	Female	5.1	22.5	80

Table 2: Islet donor characteristics for validation dataset.

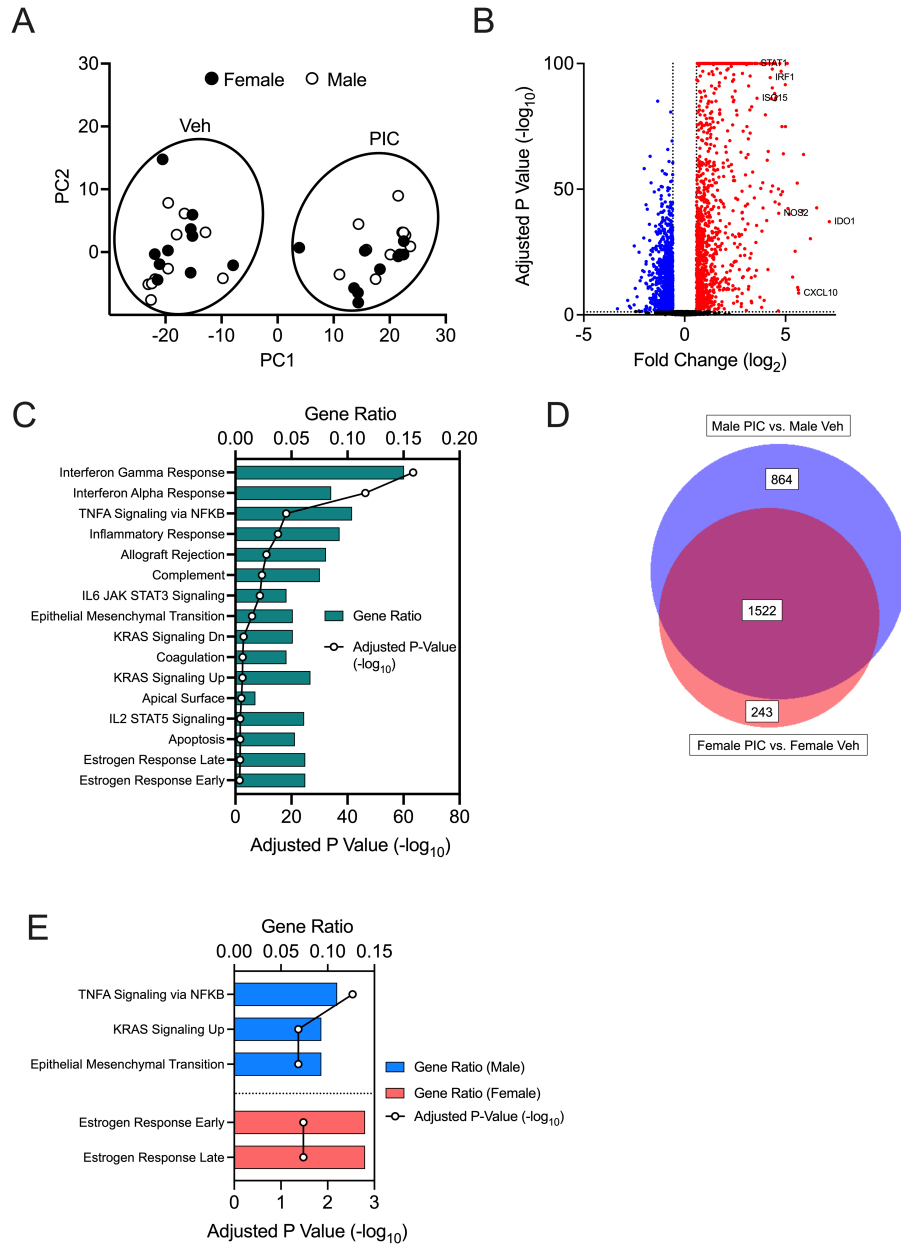


Figure 8: A validation dataset recapitulates larger proinflammatory PIC response in male islets.

(A) Multidimensional scaling plot of human islets (n=10 males, 10 females) in a novel bulk RNA sequencing data. (B) Volcano plot of all differentially expressed genes (PIC vs. Veh). Colored circles represent DEGs with fold-change ≥ 1.5 or ≤ -1.5 and an adj-p-value < 0.05 . (C) MSigDB Hallmarks pathways modulated by PIC treatment identified via overrepresentation analysis. Adj-p < 0.05 for all pathways. (D) Venn diagram of DEGs in the male and female PIC responses. (E) Overrepresentation analysis of sex-specific DEGs; MSigDB Hallmarks pathways. Adj-p-value < 0.05 for all pathways.

3.2.4 GSEA of the validation dataset recapitulates male enrichment in IFN- α response, female enrichment in developmental pathways.

Comparing the transcriptomes of PIC-treated male and female islets using GSEA reveals several signaling pathways with sex-specific enrichment (**Figure 9A**). PIC-treated male islets were enriched for IFN- α Response relative to PIC-treated females as observed in the discovery set (**Figure 7**), and also for a number of other pathways including IFN- γ Response, Unfolded Protein Response, Reactive Oxygen Species, and Apoptosis. The leading edge genes driving the male enrichment of IFN- α Response genes, notably, overlaps largely with those in the discovery set and includes genes implicated in T1D pathophysiology (e.g. *IFIH1*, *ISG15*, *IL15*, and *CASP8*) (**Figure 9B, D**). Female islets, while not enriched for the EMT pathway observed in the discovery set, were enriched in other developmental pathways including Myogenesis, Wnt Beta Catenin signaling, and Apical Junction important to the development and maintenance of endocrine cell identities in the islet^{217–221} (**Figure 9A, C, E**). These signatures further the notion that β cell identity may be more mature in male islets, and their β cell perhaps more visible to the immune system as a consequence.

Overall, RNA sequencing analysis of this novel, well-matched male and female islet RNA sequencing dataset reinforces the major findings from Section 3.1.2. In both sets, PIC-treated male islets showed a larger number of DEGs and a relative enrichment in IFN- α response genes compared to female islets, while female islets show enrichment in developmental pathways like EMT and Wnt Beta Catenin Signaling. Altogether, these data identify sexual dimorphism in the pancreatic islet transcriptome in response to inflammatory cytokines modeling the early T1D milieu, representing a potential point of divergence between sexes in the risk of T1D progression.

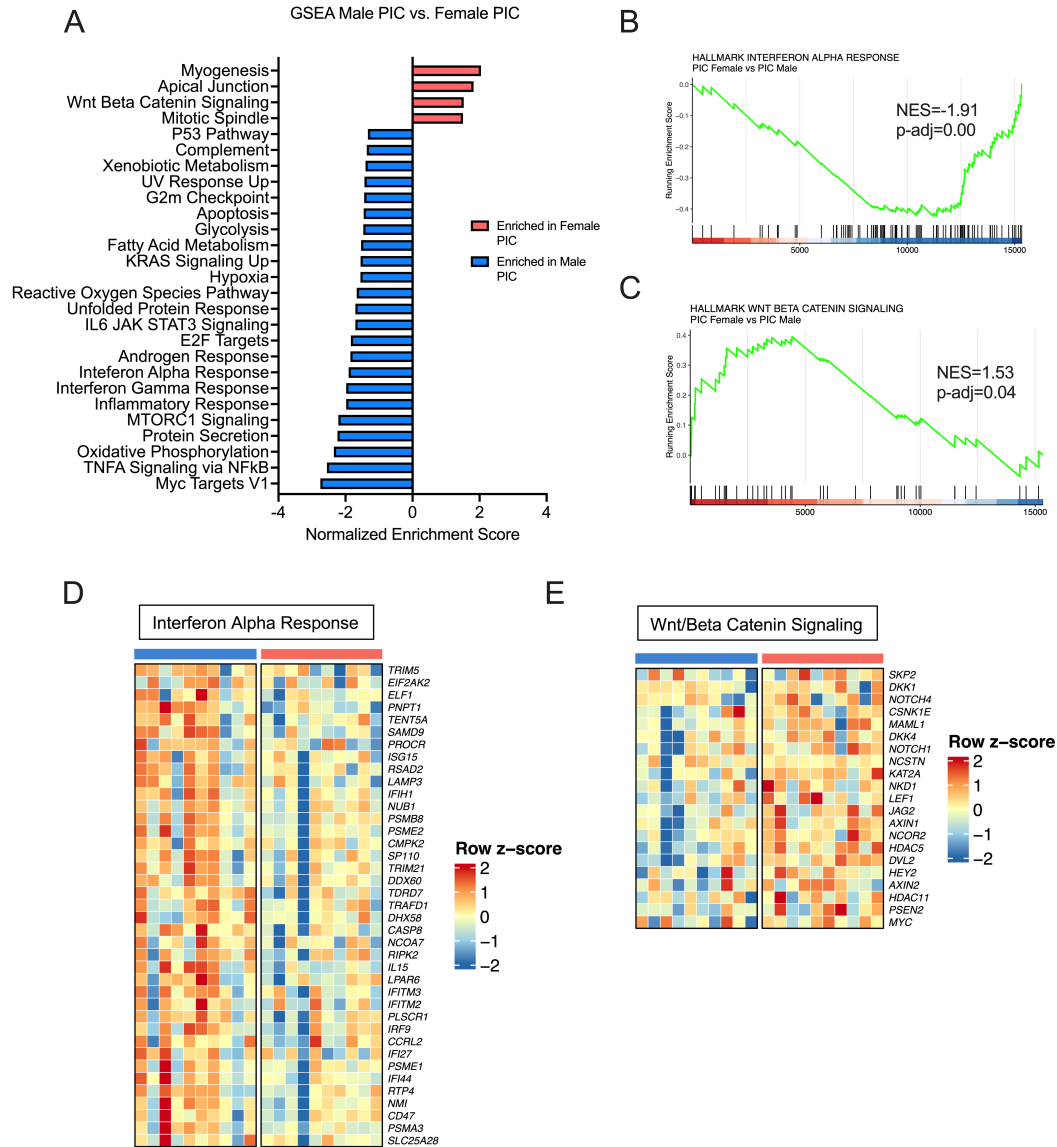


Figure 9: GSEA of the validation dataset further supports IFN- α signature in males, developmental pathway signatures in females.

(A) Hallmarks pathways significantly enriched in PIC-treated male islets (blue bars) or in PIC-treated female islets (pink bars) relative to one another by GSEA. Adj-p-value<0.05 for all pathways. (B-C) Enrichment plots for (B) Interferon Alpha Response and (C) Wnt Beta Catenin Signaling in Male PIC vs. Female PIC islets. Positive NES indicates enrichment in Female PIC, negative NES indicates enrichment in Male PIC. (D-E) Heatmaps depicting relative expression of leading edge genes for (D) Interferon Alpha Response and (E) Wnt Beta Catenin Signaling.

3.2.5 Proteomic analysis of human islets from the validation dataset also recapitulates larger male response.

After passing the human islet lysates used for the validation dataset over RNA isolation columns, I reserved the protein-containing flowthrough for mass-spectrometry based proteomics to determine whether PIC also induced sex-specific changes at the protein level. Indeed, male islets showed about 1.5 times as many unique differentially abundant proteins in response to PIC than female islets (filtered for adj-p-value<0.05 PIC vs vehicle; 498 unique to males, 338 unique to females) (**Figure 10A**). These differences reflected changes in many of the same pathways observed through the transcriptomic analyses; female specific proteomic changes involved Epithelial Mesenchymal Transition (e.g. TGFB2, TIMP3), while male specific changes related to TNF- α Signaling via NF κ B in (e.g. NF κ B1, REL, CXCL2) and Pancreas Beta Cells (e.g. PDX1, ISL1, INSM1) (**Figure 10B**). TGFB2 is important to the maintenance of β cell identity and homeostasis *in vivo*^{222,223}, and its suppression in female islets could portend loss of β cell maturity. Interestingly, several proteins related to β cell identity were also specifically downregulated in the male islets, suggesting that a loss of β cell maturity could occur in both sexes in response to PIC, though via different mechanisms. Inflammation-associated β cell dedifferentiation has been observed previously and viewed as a mechanism of β cell failure in T2D-like models²²⁴, although evidence suggests β cell dedifferentiation could be protective in an autoimmune setting^{211,212}.

Owing to the longer half-lives of proteins (vs. transcripts) and the slower kinetics of translation (vs. transcription), there may be PIC-induced proteomic changes that cannot be appreciated in these samples collected 18 hours after treatment. Overall, the larger change observed at the proteomic level in islets from male donors furthers the notion that male islets

may be preconditioned to a more aggressive response to the same inflammatory challenge compared to female islets.

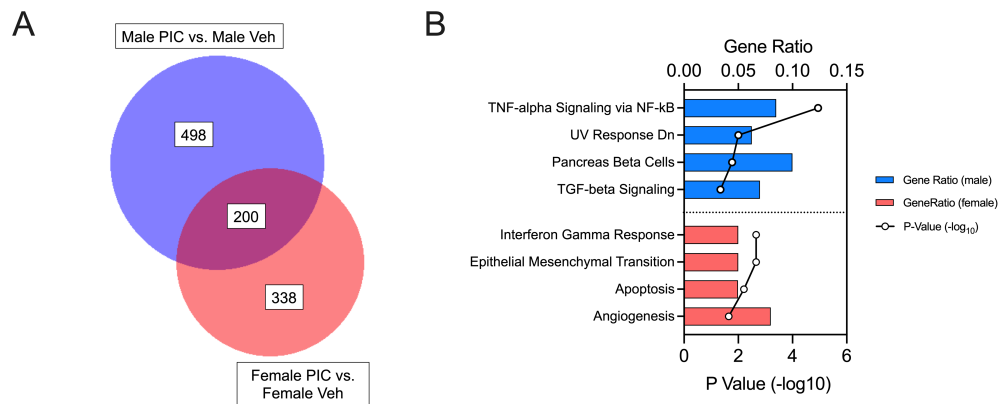


Figure 10: Proteomic analysis of the validation set recapitulates larger PIC response in males.

(A) Venn diagram representing DEGs differentially abundant in PIC vs vehicle conditions (adj-p-value<0.05) in male and female islets. (B) Overrepresentation analysis of differentially abundant proteins by sex using MSigDB Hallmarks pathways. P-value<0.05 for all pathways.

3.2.6 Human T1D β cells show suppressed EMT signature, elevated IFN- α signature compared to AAB+ and nondiabetic islets

The Human Pancreatic Analysis Program's (HPAP) PANC-DB is a publicly accessible data repository containing genomic and functional data from islets of nondiabetic, AAB+, and donors with T1D, or T2D^{177,225,226}. I utilized the available single cell RNA sequencing (scRNAseq) data from nondiabetic, AAB+ and T1D donors (**Table 3**), subsetting out the β cells specifically to perform GSEA comparisons. By GSEA, IFN- α signaling was enriched in both AAB+ and T1D β cells compared to non diabetic β cells, and also in T1D β cells compared to AAB+ β cells (**Figure 11**). The leading edge genes driving IFN- α enrichment in both AAB+ vs. nondiabetic and T1D vs. AAB+ β cells overlapped well with genes driving enrichment in male islets compared to female islets in the earlier analyses (e.g. *IFIH1*, *IL15*, *CASP8*). This increase in β cell IFN- α response by stage of T1D progression further supports the notion that enrichment of this pathway in male islets might increase β cells immunogenicity and contribute to higher male T1D incidence. Conversely, EMT genes were enriched in nondiabetic β cells compared to both AAB+ and T1D β cells and in AAB+ β cells relative to T1D β cells (**Figure 11**). This loss of EMT genes with T1D progression could indicate that this pathway- observed to be enriched in female islets in the discovery dataset (**Figure 7**)- is protective against disease development.

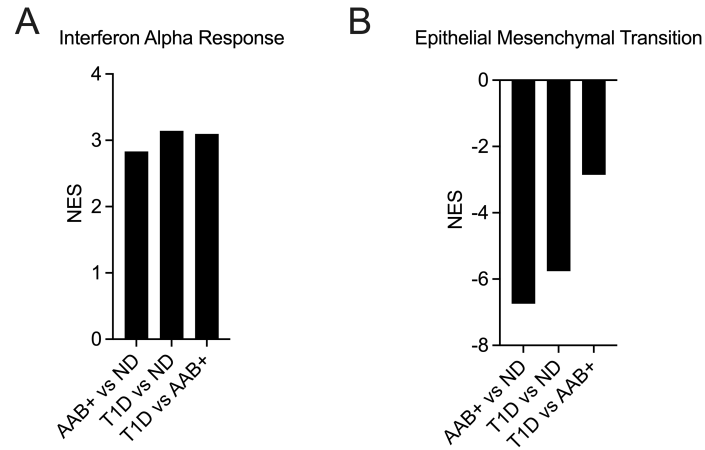


Figure 11: IFN- α enrichment (and EMT suppression) in human T1D and AAB+ β cells compared to nondiabetic controls.

GSEA performed on the β cells the HPAP dataset comparing AAB+ vs nondiabetic (ND) donors, T1D vs ND donors, and T1D vs AAB+ donors utilizing the MSigDB Hallmarks pathways. (A) Interferon Alpha Response and (B) Epithelial Mesenchymal Transition. Adj-p-value<0.05 for all comparisons.

Donor ID	Donor type	Age (yrs)	Sex	BMI	HbA1C
RRID: SAMN19776470	Non-diabetic	5	F	16.3	6.8
RRID: SAMN19776475		3	F	12	5.3
RRID: SAMN22562815		4	M	20.63	4.9
RRID: SAMN19776478		8	M	16.82	N/A
RRID: SAMN19776465		13	M	18.6	5.2
RRID: SAMN19776467		23	F	16	5.2
RRID: SAMN19776457		24	M	20.8	4.9
RRID: SAMN19842611		25	M	23.96	5.6
RRID: SAMN22562810		28	F	24.7	5
RRID: SAMN19776458		31	F	32.71	4.4
RRID: SAMN19776466		35	M	26.91	5.2
RRID: SAMN19842585		33	M	32.89	5.6
RRID: SAMN19776468		35	F	21.9	5.3
RRID: SAMN19776471		M	35	23.98	5.4
RRID: SAMN19776453		F	39	34.7	4.7
RRID: SAMN19776455	Donors with single islet-specific autoantibody (AAb1+)	M	18	24.3	5.5
RRID: SAMN19776460		M	23	28.6	5.3
RRID: SAMN19776469		M	13	18.34	5.7
RRID: SAMN19776476		F	27	26.2	5.2
RRID: SAMN19776480		M	29	37.2	5.4
RRID: SAMN19776481		F	21	28.99	5.1
RRID: SAMN19842601		M	19	23.1	5.6
RRID: SAMN19842621		M	21	25.59	5.6
RRID: SAMN19776474	Donors with single islet-specific autoantibody (AAb2+)	M	15	24.07	5.9
RRID: SAMN25600003		M	15	23.59	5.3
RRID: SAMN19776451	Donors with T1D	M	14	13.2	N/A
RRID: SAMN19776452		F	13	21.4	N/A
RRID: SAMN19776454		F	17	21.35	8.9
RRID: SAMN19776463		F	10	16.3	9
RRID: SAMN19776485		M	24	27.9	10.4
RRID: SAMN19842593		M	24	16.98	13
RRID: SAMN19842600		F	12	15.42	9.8
RRID: SAMN19842613		F	12	18.5	13.3
RRID: SAMN19842616		F	15	19.3	10.4

Table 3: Donor characteristics for samples used from HPAP database.

SOURCE: Muralidharan et al, *J Clin Invest* (2024)²²⁷. Open Access.

3.3 Discussion

In this chapter, I leveraged publicly available and novel bulk RNA sequencing datasets of human islets challenged with IL-1 β and IFN- γ to determine whether sex influences islet response to these diabetogenic inflammatory signals. It is increasingly evident that intrinsic β cell ‘fragility’- susceptibility to damage and death in the face of endogenous (i.e. insulin biosynthesis) or external pressures like viral infection and inflammation- plays a role in whether islet autoimmunity develops and progresses to T1D^{100–104}. In humans with T1D, a subset of β cells appear to be resilient to autoimmune attack; insulin+ cells expressing immune inhibitory signal PD-L1, for example, are still present in the islets of some people with T1D years after onset²²⁸. The broader proinflammatory response to an identical cytokine challenge in male islets relative to female islets observed here, illustrated by their larger number of PIC-induced DEGs (**Figure 6; Figure 8**) and enrichment in IFN- α response genes (**Figure 7; Figure 9**), as well as pathways like Unfolded Protein Response and Reactive Oxygen Species (**Figure 9**), may indicate increased β cell fragility in male islets.

There are several mechanisms through which IFN- α signaling increases β cell dysfunction and visibility to cytotoxic immune cells. In human β cells, IFN- α exposure increases ER stress and production of neoantigens as well as their presentation on hyperexpressed HLA I complexes^{229,230}. This IFN- α -induced HLA I hyperexpression persists long after initial cytokine exposure, and CD8+ T cells in T1D islets have been shown to be reactive against HLA-bound β cell neoantigens^{129,197}. Further illustrating the importance of HLA I expression in immune-mediated killing of β cells, ‘hypoimmune’ human islet cells engineered to lack HLA I expression escape immune surveillance when transplanted into immunocompetent mice²³¹.

T1D candidate gene *IFIH1*, as well as NK- and CD8+ T cell-activating cytokine gene *IL15* and proapoptotic protease gene *CASP8*, were on the leading edge of male islet enrichment

of IFN- α response in both datasets analyzed in Section 3.2.1 and 3.2.2 (**Figure 7; Figure 9**). Demonstrating relevance to risk of T1D progression, these same genes helped to drive the enrichment of IFN- α response in human β cells in T1D and AAB+ β cells compared to nondiabetic β cells (**Figure 11**). MDA5 (encoded by the *IFIH1* gene) is a double-stranded RNA sensor whose activity stimulates proinflammatory transcription factors (e.g. IRF3 and NF- κ B), HLA I expression, and secretion of type I interferons and chemokine CXCL10 by the β cell itself²²⁹. Several SNPs in *IFIH1* are associated with T1D risk²³², and reduced *IFIH1* expression protects from diabetes development in mouse models of T1D^{233,234}. IL-15 is a strong inducer of NK cell IFN- γ production and of effector memory function in CD8+ T cells, producing a pool of cytotoxic and often tissue-resident effectors that have been observed human insulinitis lesions^{205,235,236}. Elevated serum IL-15 levels have been measured in people with T1D²⁰⁴. CASP8 is a proapoptotic protease that activates executioner caspases CASP-3 or -7 downstream of extrinsic apoptotic signals (i.e. binding of death receptors like TRAIL or FAS), and β cell specific depletion of *CASP8* protects against immune-mediated death in the STZ model of T1D²⁰¹. Altogether, the exaggerated IFN- α response signature observed in male islets in both RNA sequencing datasets could *in vivo* exacerbate immune infiltration of the islets, β cell visibility to the immune system, and β cell-directed cytotoxicity.

Across two human islet RNAseq datasets, developmental pathway genes were enriched in female islets compared to male islets (**Figure 7; Figure 9**). HPAP data reveals that EMT may be suppressed in the β cell during T1D progression (**Figure 11**). A role for EMT in adult islet physiology or in T1D pathogenesis has not been clearly defined. EMT has long been associated with organogenesis, wound healing and tissue remodeling, and cancer progression²⁰⁶. Interestingly, EMT helps to drive progression of multiple cancers by facilitating cancer cell

evasion of immune surveillance^{237,238}. In the islet, activation of both EMT and Wnt genes have been associated with a ‘dedifferentiated’ β cell phenotype characterized by suppressed expression of identity genes like *Pdx1*^{207,208,210,217,220}. While regarded as a form of β cell failure in T2D^{21,22}, β cell dedifferentiation has been shown to protect β cell survival in models of T1D^{211,212}. For example, a population of β cells expressing lower levels of β cell identity genes (e.g. *Pdx1*, *Ins1/2*, *Nkx6-1*, *MafA*) and more PD-L1 develops with age in NOD mice and survives the onslaught of autoimmunity²¹². Whether the enriched developmental pathway signatures in female islets in these datasets might signify less mature β cell identity relative to males will require further investigation.

Overall, the work presented in this chapter demonstrates sexual dimorphism in the human pancreatic islet transcriptome, both at baseline and in response to T1D-associated proinflammatory cytokines. In response to the same PIC stimulus, male islets show more differentially expressed genes and an enriched signature of IFN- α response, a key trigger for T1D progression also enriched in the β cells of individuals with T1D. Female islets show enriched developmental pathway signatures, a feature shared by β cells from nondiabetic donors compared to AAB+ and T1D donors. Through what mechanism sex might precondition these dimorphic responses requires additional investigation. The genes driving sex-specific enrichment of IFN- α and EMT pathways are by and large autosomal, suggesting that these dimorphisms cannot be explained simply by differences in sex chromosome complement. Interestingly, in the validation dataset, DEGs unique to females enriched two Estrogen Response pathways. In the next chapter, I will explore whether the sex hormone estradiol, shown to protect β cell health and function across multiple contexts, might drive sex differences in islet phenotype, response to inflammation, and susceptibility to autoimmunity.

CHAPTER 4: ESTRADIOL SUPPRESSES ISLET INFLAMMATORY SIGNALING AND PROTECTS AGAINST DIABETES DEVELOPMENT IN NOD MICE

4.1 Introduction

A remarkable 80% of the millions of Americans living with autoimmune diseases are female (46, XX), and many autoimmune diseases (e.g. Sjögrens, lupus, multiple sclerosis) show large female predominances^{131,132,239,240}. Incomplete inactivation of immune-related genes on the X chromosome as well as proinflammatory effects of estradiol on immune cells have been suggested to contribute to the more robust immune responses observed in females, which may protect from infections and cancers while predisposing to autoimmunity^{135,137,141,241,242}. These ideas stand in contradiction, however, with the relative protection of females from an autoimmune disease like T1D^{144–147}.

The influence of sex hormone signaling, particularly that of estradiol (E2), on immune cell function in autoimmunity has been studied extensively with variable results. E2 is the predominant circulating estrogen during reproductive years and bears the highest affinity for known estrogen receptors ER α , ER β , and GPER1. Interestingly, E2 has proven capable of skewing immune cells toward pro- or anti-inflammatory phenotypes depending on cell type, dosage, or disease context^{141,243}. For example, E2 is considered an exacerbating factor in systemic lupus erythematosus (SLE), wherein E2 is suspected of sustaining autoantibody-producing B cell populations and enhancing type 1 IFN production by plasmacytoid dendritic cells (pDCs)^{243–246}. In sharp contrast, E2 and pregnancy hormone estriol (E3) have been proposed as treatment modalities for multiple sclerosis (MS)^{247–251}. Women with MS experience lower rates of relapse during pregnancy, a period of high E2 and E3²⁴⁸. Estrogens in mouse models of MS (and pregnancy in humans) have been shown to skew T cell responses away from inflammatory Th17 and Th1 toward more anti-inflammatory Th2 like phenotypes, while other

studies suggest that E2 signaling has protective effects directly at the target tissue (CNS), enhancing endogenous myelination and decreasing immune cell recruitment^{252–255}.

While the male sex bias of T1D is increasingly appreciated, its mechanistic underpinnings are largely unexplored. Male and female incidence of T1D diverges after the age of puberty¹⁴⁴, pointing to sex hormones as logical suspects for protection from or predisposition to disease. Exogenous E2 has been shown to protect female NOD mice from autoimmune diabetes development, in part through activation of invariant natural killer T (iNKT) cells¹⁶². E2 is known to boost glucose stimulated insulin secretion and to protect β cells from death under glucolipotoxic, oxidative, and inflammatory stresses^{154,159,160,256}, but whether E2 signaling at the islet plays a role in protection from T1D *in vivo* is unknown. In this chapter, I utilize acute *in vitro* E2 treatment of β cells and islets, chronic exogenous E2 treatment of male and female NOD mice, and islet single cell RNA sequencing (scRNAseq) to explore the hypothesis that E2 signaling in the islet microenvironment decreases β cell immunogenicity, protecting against cytotoxicity and diabetes development.

4.2 Results

4.2.1 PIC increases the expression of *Esr1* in β cells and islets.

The role of E2 in inflammatory signaling varies widely across cell types and disease contexts^{141,243,257,258}. Stimulation of islets with E2 protects against apoptosis in inflammatory settings¹⁶⁰, while depletion of ER α sensitizes the islet to death and destruction *in vitro* and *in vivo*^{158,256}. To investigate whether the E2 signaling pathway is modulated by PIC, such as those present in the T1D islet milieu, I treated the murine insulinoma 6 (MIN6) β cell line with PIC (TNF- α , IL-1 β , IFN- γ) and measured expression of *Esr1* and *Esr2* (encoding ER α and ER β ,

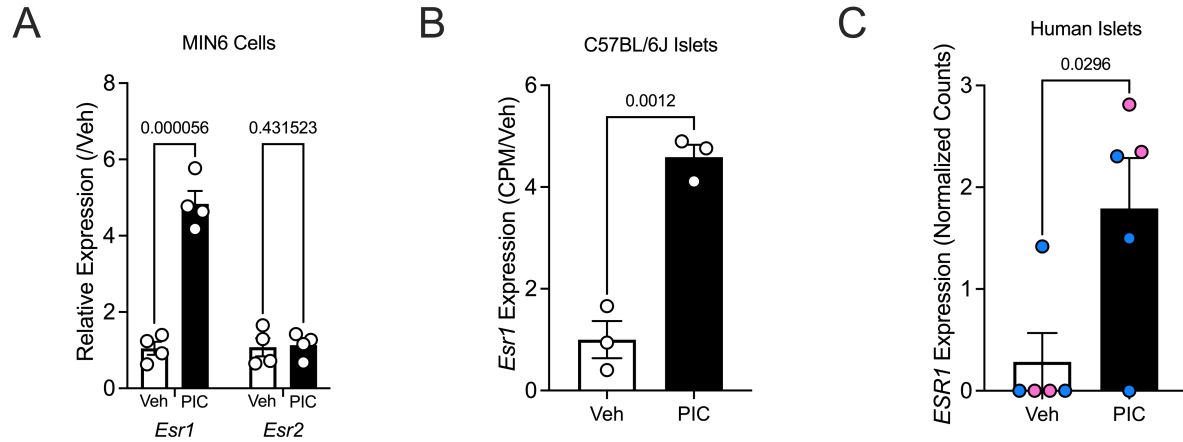


Figure 12: PIC increase *Esr1* expression in mouse and human islet cells.

(A) Relative expression of *Esr1* and *Esr2* in MIN6 cells treated with vehicle or PIC (TNF- α , IL-1 β , IFN- γ) for 18 hours measured via qRT-PCR. (B) *Esr1* expression in C57BL/6 mouse islets treated with vehicle or PIC (TNF- α , IL-1 β , IFN- γ) for 24 hours; data extracted from published bulk RNA sequencing¹⁷⁶. (C) *Esr1* expression in human islets treated with vehicle or PIC (IL-1 β , IFN- γ) for 24 hours; data extracted from published bulk RNA sequencing¹⁷³. Blue dots represent samples from male islet donors, pink dots represent samples from female islet donors. Statistical comparisons presented are p-values calculated with student's t-test.

respectively) via quantitative RT-PCR (qRT-PCR). *Esr1* expression was significantly increased with PIC, while *Esr2* levels were unchanged (**Figure 12A**). I next compared *Esr1* expression in C57BL/6J mouse islets as well as human islets before and after PIC treatment in published bulk RNA sequencing datasets^{173,176}. In both these contexts, PIC again increased *Esr1* expression (**Figure 12B-C**). Together, these data suggest that islets may increase their receptiveness to E2 signaling, shown to be protective of islet function and survival^{158,160,256}, in the face of inflammation.

4.2.2 E2 decreases expression of β cell identity genes while increasing expression of developmental pathway genes.

To assess β cell responsiveness to E2 *in vitro*, I treated MIN6 cells with E2 concentrations ranging from 10 nM to 500 nM and measured expression of selected ER α target genes: *Esr1* itself, *Greb1* (encoding an ER α cofactor), and *Pgr* (encoding progesterone receptor). Expression of all 3 genes was significantly increased at all concentrations tested after 18 hours (**Figure 13A-C**). When I then treated MIN6 for up to 48 hours with the lowest concentration (10 nM E2), I observed that prolonged E2 treatment produced more pronounced increases in ER α response gene expression (**Figure 13D-F**). Because of the enriched developmental pathway signatures observed in female islets (**Figure 7; Figure 9**), I hypothesized that E2 exposure might alter the state of β cell maturity. Over the 48-hour time course, expression of β cell maturity gene *Ucn3* declined while expression of *Hes1*, an effector of Notch signaling associated with β cell dedifferentiation²⁵⁹, increased (**Figure 14A-B**). Interestingly, islets from male C57BL/6J mice treated with E2 overnight showed decreased expression of β cell identity gene *Pdx1* (**Figure 14C**), whose activity has been suggested to repress developmental signaling in the β cell under physiologic conditions²⁶⁰. Overall, these data suggest that β cells respond to E2 treatment via

canonical ER α pathways, and that E2 may decrease β cell maturity while activating developmental pathways like those I observed to be enriched in female human islets relative to male islets (**Figure 7; Figure 9; Figure 11**).

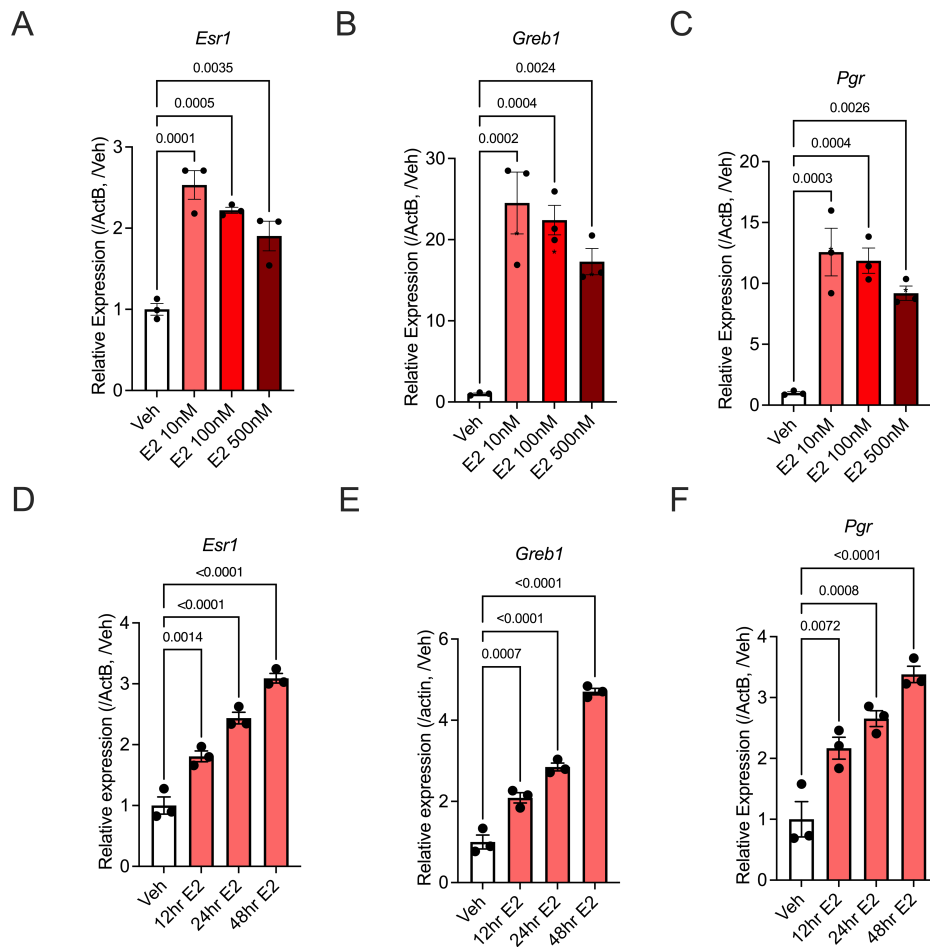


Figure 13: β cells increase expression of canonical ER α targets in response to E2. (A-C) Gene expression measured in MIN6 cells treated for 18 hours with vehicle or E2 (10nM, 100nM, or 500nM) via qRT-PCR. (D-F) Gene expression measured in MIN6 cells treated with vehicle or 10 nM E2 for 12, 24, or 48 hours via qRT-PCR.

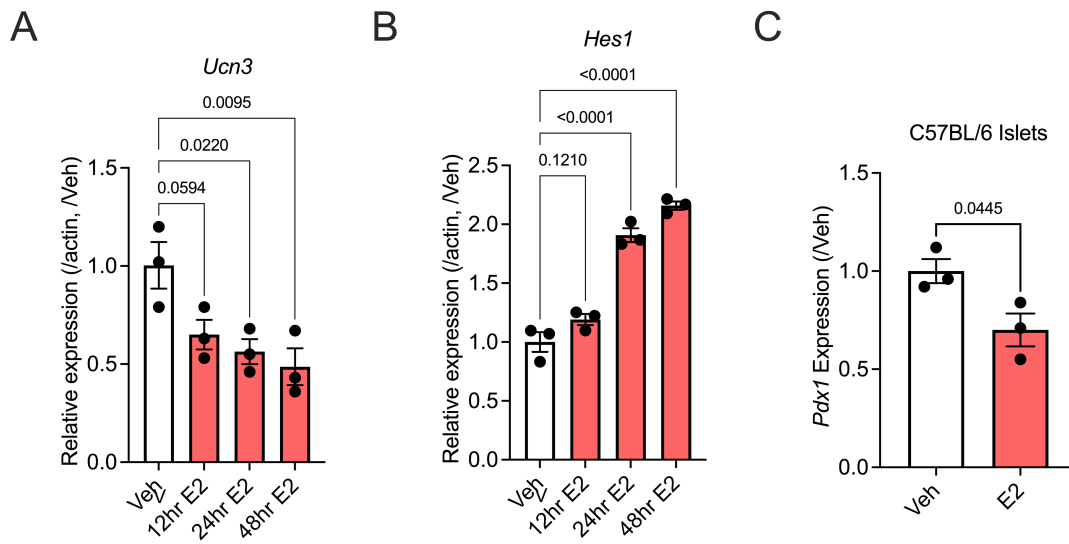


Figure 14: E2 treatment decreases expression of β cell maturity genes while activating developmental pathways.

(A-B) Gene expression measured in MIN6 cells treated with vehicle or 10 nM E2 for 12, 24, or 48 hours via qRT-PCR. (C) Gene expression measured in islets isolated from male C57BL/6J mice and treated with vehicle or 10nM E2 for 18 hours via qRT-PCR.

4.2.3 Human islets show sexually dimorphic response to estradiol treatment at the transcriptomic and proteomic levels.

I next sought to investigate the direct impact of E2 signaling on human islets. A subset of islets from each human donor in the ‘validation set’ cohort described in Chapter 3 (**Table 2**; **Figure 8**) were treated for 18 hours with 10 nM E2, after which both RNA and protein were collected. The RNA from the E2-treated samples was bulk sequenced alongside the vehicle and PIC treated samples discussed earlier. The effects of this acute E2 treatment were much more subtle than those of acute PIC; thus, for this analysis I used the looser filtering criteria of fold-change ≥ 1.25 or ≤ -1.25 and p-value < 0.05 . Taken separately by sex, the transcriptomic responses to E2 were starkly different. While males showed 235 DEGs in response to E2, females showed 171 DEGs, with only 17 genes overlapping between sexes (**Figure 15A-C**). Among the overlapping response genes was canonical ER α target *GREB1* (**Figure 15A-B**). Overrepresented in the males was the Allograft Rejection pathway, including proinflammatory genes *IL2RB* and *IFI44L* which were downregulated by E2, as well as Estrogen Response Late and EMT (**Figure 15A,D**). Overrepresented in the female islets were pathways including Estrogen Response Early and Interferon Gamma Response; proinflammatory genes *IDO1* and *CXCL11* were downregulated by E2, for example (**Figure 15B,D**). While the individual DEGs were highly disparate between sexes, GSEA revealed some functional overlap in the E2 responses. Intriguingly, E2 treatment suppressed the Interferon Gamma Response pathway in islets from both sexes (**Figure 15E-F**), suggesting that prior exposure to E2 *in vivo* may indeed help to explain the diminished IFN signatures observed in female islets compared to male islets (**Figure 7**; **Figure 9**).

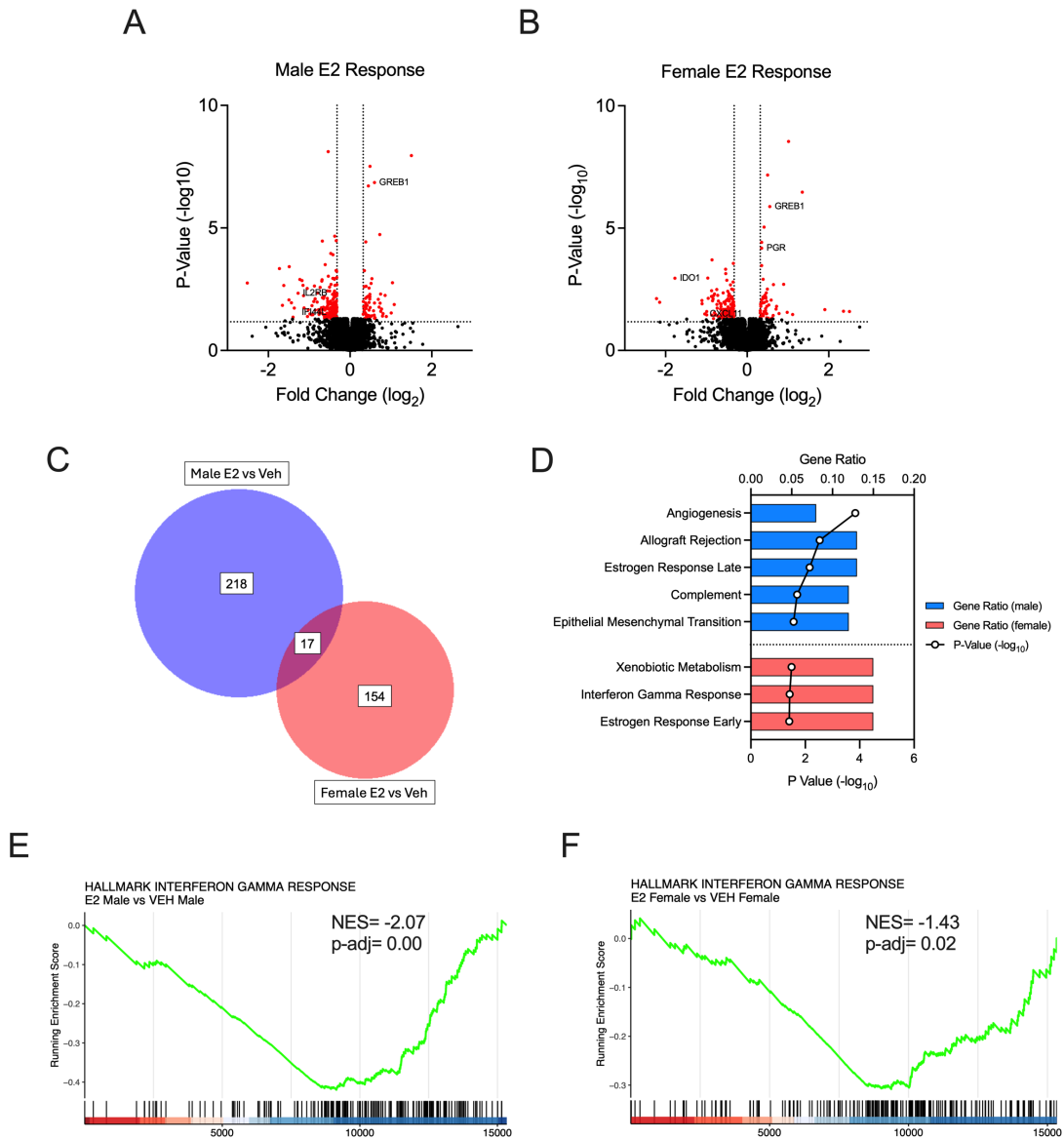


Figure 15: Human islets show a sexually dimorphic transcriptomic response to E2.

(A-B) Volcano plots of differentially expressed genes (DEGs) in human islets after treatment 10nM E2 for 18 hours, measured via bulk RNA sequencing. Red dots represent genes with >1.25 or <-1.25 fold change and $p\text{-value} < 0.05$ between treatment groups. (C) Venn diagram representing DEGs in response to E2 separated by sex of the islet donor. (D) Overrepresentation analysis of E2 response genes in islets from male and female donors; MSigDB Hallmarks Pathways. $P\text{-value} < 0.05$ for all pathways. (E-F) GSEAs plot of Hallmarks Interferon Gamma Response pathway in (E) male E2 vs vehicle and (F) female E2 vs vehicle. Positive NES signifies enrichment in E2, negative NES signifies enrichment in vehicle.

Utilizing mass-spectrometry based proteomics, I observed that the E2 response was also highly sexually dimorphic at the protein level in these samples (**Figure 16A**). Here, there was little overlap between sexes of individual proteins responding to E2 and also little functional overlap. Male islets showed changes in apoptotic signaling pathways (e.g. Apoptotic Execution Phase) in response to E2 (**Figure 16B**), a signal which was not detectable in the female response. Instead, female changes included several metabolic pathways and, of note, Plasma Membrane Estrogen Receptor Signaling (**Figure 16B**).

In summary, the estrogen-stimulated β cell and islet data *in vitro* suggest that E2 incites potentially protective signaling in both male and female islets, such as downregulating proapoptotic signals in males and decreasing IFN- γ signaling in both sexes. The overall lack of overlap between differentially expressed genes and proteins in response to E2 by sex might reflect different modalities of E2 signaling, perhaps driven by E2 sensing by different receptor types (i.e. ER α vs. ER β isoforms, or nuclear vs. membrane-associated receptor pools) or by differential accessibility of estrogen response elements on the DNA between sexes. These data raise the possibility that E2-stimulated signaling *in the islet cells* may contribute to protection from autoimmune destruction.

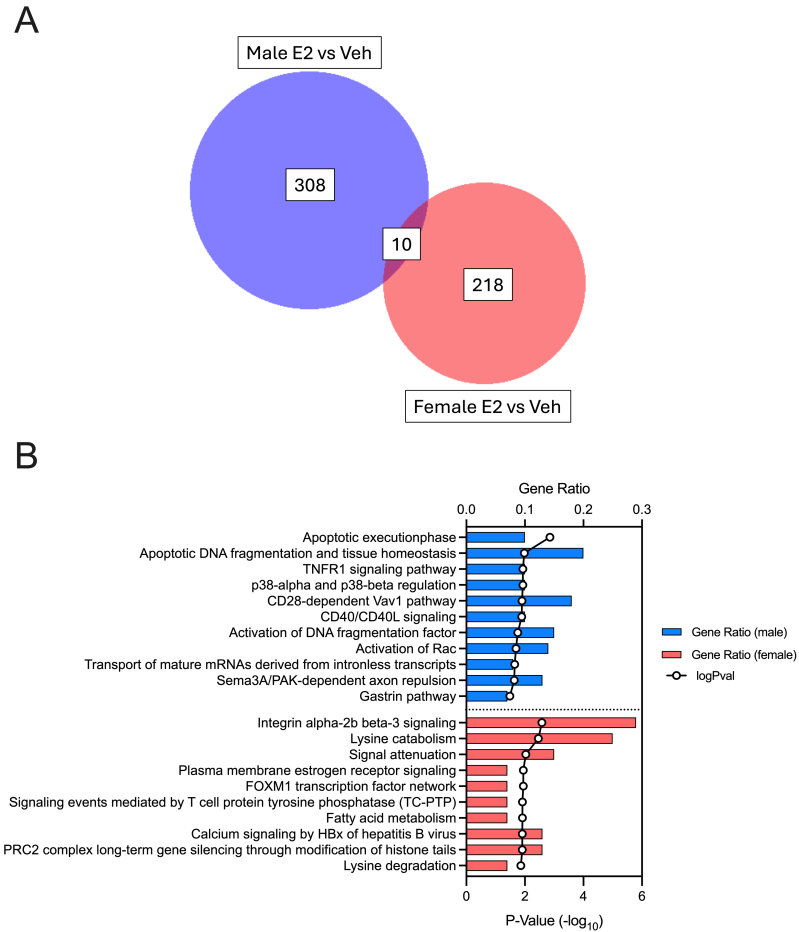


Figure 16: Human islets show a sexually dimorphic proteomic response to E2.

(A) Venn diagram representing differentially abundant proteins upon 18 hours of treatment with 10nM E2 in human islets from male and female donors, measured via mass-spectrometry based proteomics. Proteins filtered for adjusted p-value<0.05. (B) Overrepresentation analysis of differentially abundant proteins with E2 treatment in islets from male or female donors; BioPlanet 2019 pathways. P-value<0.05 for all pathways.

4.2.4 NOD islets lack PIC-induced *Esr1* and *Ar* responses.

The non-obese diabetic (NOD) mouse strain was developed in Japan in the 1970s by selective inbreeding after Makino and colleagues observed a female mouse that spontaneously developed polyuria, glycosuria, and rapid weight loss²⁶¹. The rapid onset autoimmune diabetes observed in NOD mice shares many characteristics with human T1D, including genetic susceptibility largely driven by MHC class I and II, islet autoantibody development, elevated proinflammatory cytokines, and immune infiltration of the islet by autoreactive lymphocytes^{262,263}. Notably, unlike in human T1D, female NOD mice typically show higher diabetes incidence rates than male NOD mice, a phenomenon that has been attributed to differences in microbiome composition and epistatic effects of sex on susceptibility locus *Idd4*^{264–268}. Despite this, prior studies have shown that delivery of exogenous estradiol and other ‘pregnancy hormones’ actually protects female NOD mice from diabetes development^{162,269}. These studies primarily investigated the effect of exogenous estradiol on immune cells, in particular regulatory iNKT cells¹⁶².

To determine whether NOD mice show the same upregulation of E2 receptiveness via ER α that I observed in other mouse and human islet cells in the face of inflammation (**Figure 12**), I isolated islets from male and female C57BL/6J and NOD islets and treated them with PIC (TNF- α , IL-1 β , IFN- γ). Islets from both male and female C57BL/6J mice increased *Esr1* expression in response to PIC and reciprocally decreased their expression of *Ar* (encoding the androgen receptor) (**Figure 17**). By striking contrast, islets from NOD mice lacked both of these responses (**Figure 17**). Alongside the protection of NOD mice from diabetes with E2 treatment observed in past studies¹⁶², these data raise the possibility that NOD mice are made more

vulnerable to diabetes by their lack of islet E2 signaling responses during the inflammatory challenge of insulinitis *in vivo*.

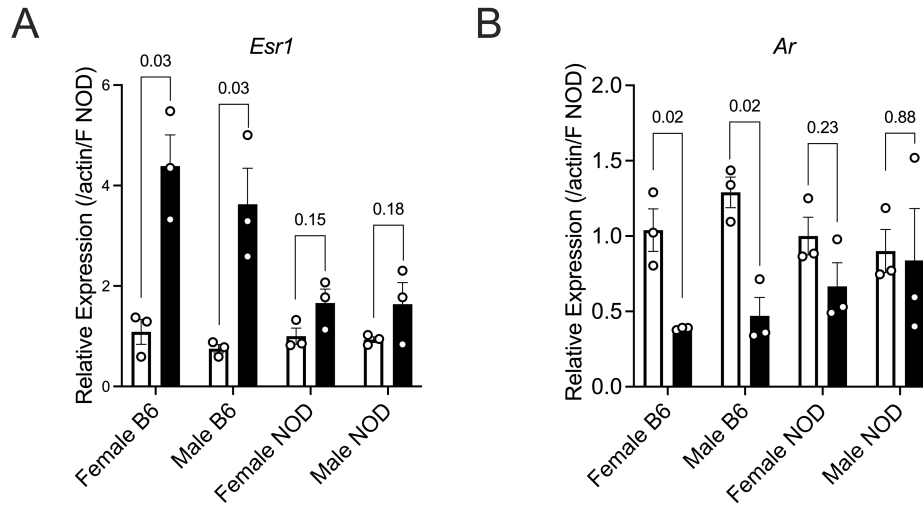


Figure 17: NOD mice lack *Esr1* and *Ar* responses to PIC observed in C57BL/6.

Gene expression measured via qRT-PCR in islets isolated from 8 week old male and female C57BL/6 and male and female NOD mice, treated with vehicle or with PIC (TNF- α , IL-1 β , IFN- γ) for 18 hours. Statistics shown are unpaired student's t-test.

4.2.5 Exogenous estradiol protects against diabetes development in male and female NOD mice.

Prior studies suggest that chronic E2 treatment protects against autoimmune diabetes development in female NOD mice^{162,269}. To investigate if exogenous E2 might exert protective effects on the islet itself, as well as whether such protection would extend to male NOD mice, I treated male and female NOD mice with placebo or subcutaneous 0.5 mg E2 pellets formulated to deliver their contents over 90 days. I inserted the implant at the age of 6-weeks, a time at which NOD mice begin to exhibit insulinitis but do not yet develop diabetes²⁷⁰, and followed the mice for diabetes incidence (2 consecutive blood glucose readings over 250 mg/dl) out to 25 weeks of age (**Figure 18A**). In line with prior work, female mice implanted with E2 were completely protected from diabetes onset during the 90 days of pellet activity, while about 50% of placebo treated females developed diabetes during the same period (**Figure 18B**). 25% of E2 treated females went on to develop diabetes after the expiration of the E2 pellet. Interestingly, E2-treated male mice were completely protected from diabetes during the entire study period (**Figure 18C**).

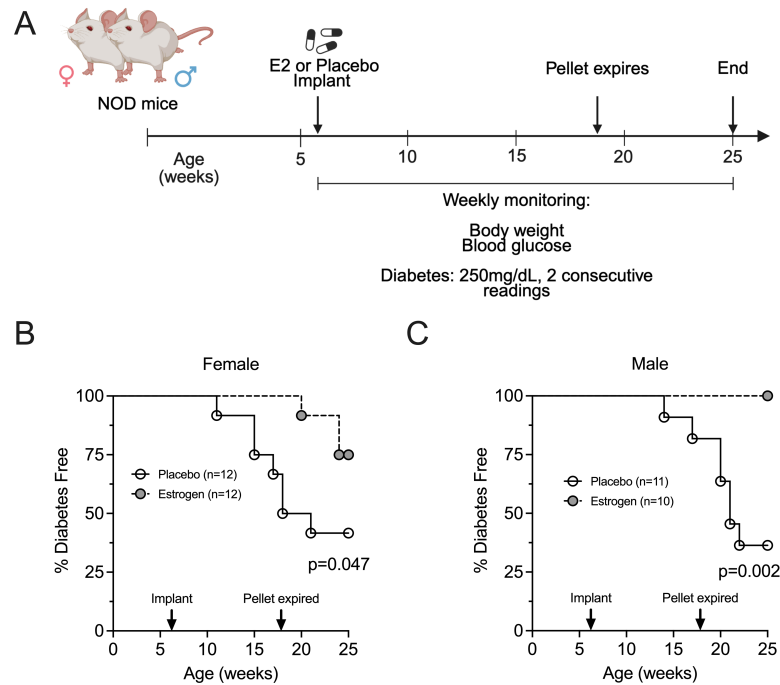


Figure 18: E2 protects male and female NOD mice from diabetes development.

(A) Schematic of study design; NOD mice were implanted with E2 or placebo implants at 6 weeks of age and monitored for diabetes development until 25 weeks of age. Created with BioRender.com. (B) Kaplan-Meier curves showing diabetes incidence by treatment group in female NOD mice. N=12 biological replicates across 2 experiments. (C) Kaplan-Meier curves showing diabetes incidence by treatment group in male NOD mice. N=10-11 biological replicates across 2 experiments. P-value reflects Mantel-Cox test between treatment groups.

4.2.6 Estradiol treatment does not change β cell mass but does decrease severe insulitis after 4 weeks.

To investigate the mechanism of E2 protection against diabetes development in the NOD mouse, I next performed a series of experiments treating NOD mice with the same E2 or placebo pellets during the prediabetic period (6-10 weeks). I collected serum and pancreata at 10 weeks of age to examine the impacts of E2 treatment before the age of typical diabetes onset (~12 weeks) (**Figure 19A**). Serum E2 levels were confirmed to be higher at the time of euthanasia as measured by ELISA (**Figure 19B**). Not unexpectedly, E2 treatment did not change average β cell mass after 4 weeks in either male or female mice during this “pre-diabetic” phase (**Figure 19C**). Notably, whereas E2 treatment did not prevent the infiltration of islets by immune cells, it did decrease the average proportion of severely insulitic islets, defined as those in which immune infiltrates cover 50% or greater of islet area (scores 3 and 4) (**Figure 19D**). These results suggest that E2 does not protect from diabetes development by totally precluding immune infiltration of the islet, but perhaps instead by changing the nature of the interaction between β cells and immune cells once they arrive. I next sought to investigate the phenotypes of islet endocrine and immune cells in placebo and E2 treated mice via scRNAseq.

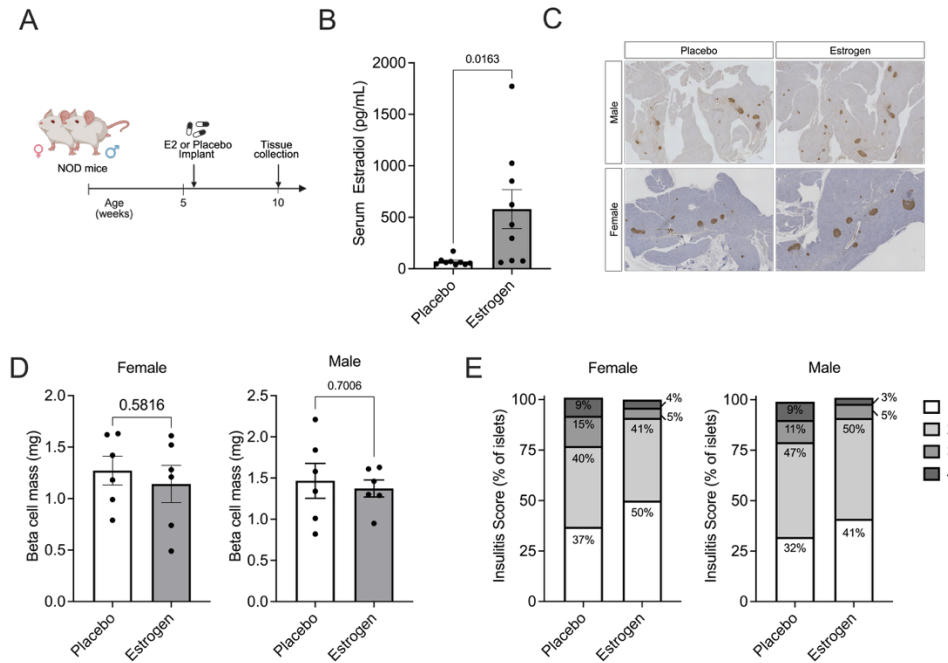


Figure 19: E2 treatment decreases insulinitis severity after 4 weeks.

(A) Schematic of treatment. Female and male NOD mice were treated with placebo or E2 pellets for 4 weeks before tissue collections. Created with BioRender.com. (B) Serum E2 levels measured by ELISA (N=9 per treatment group). (C) Representative IHC images of female and male pancreata stained for insulin by treatment group. (D) β cell mass measured in female and male mice by treatment group. (E) Insulinitis scoring in female and male mice by treatment group. N=6 mice per treatment group, 3 pancreas slides scored per mouse for both β cell mass and insulinitis. Statistics shown are student's unpaired t-test.

4.2.7 Estradiol does not change the proportion of major cell types in the islet after 4 weeks by scRNAseq.

To assess the quantities and transcriptional profiles of islet endocrine and immune cells after treatment of prediabetic NOD mice with placebo or E2 pellets, I next performed scRNAseq on islets isolated from female NOD mice after 4 weeks of treatment. 3 samples were sequenced per treatment group, each comprised of islets pooled from 2 animals. I first visualized the transcriptional relationships between cells in this dataset using uniform manifold approximation projection (UMAP); using the marker genes for each cell cluster, I identified all major islet endocrine cell types (β , α , δ , and PP cells), infiltrating immune cells (T and B lymphocytes, Macrophages), as well as some supporting cell types (acinar, ductal, and mesenchymal cells, erythrocytes) (**Figure 20A**). There was no statistical difference in the proportion of any of these major cell types making up the placebo vs. E2-treated islets (**Figure 20B**). I next utilized differential expression analyses to interrogate the transcriptional states of endocrine and immune cell types between treatment groups.

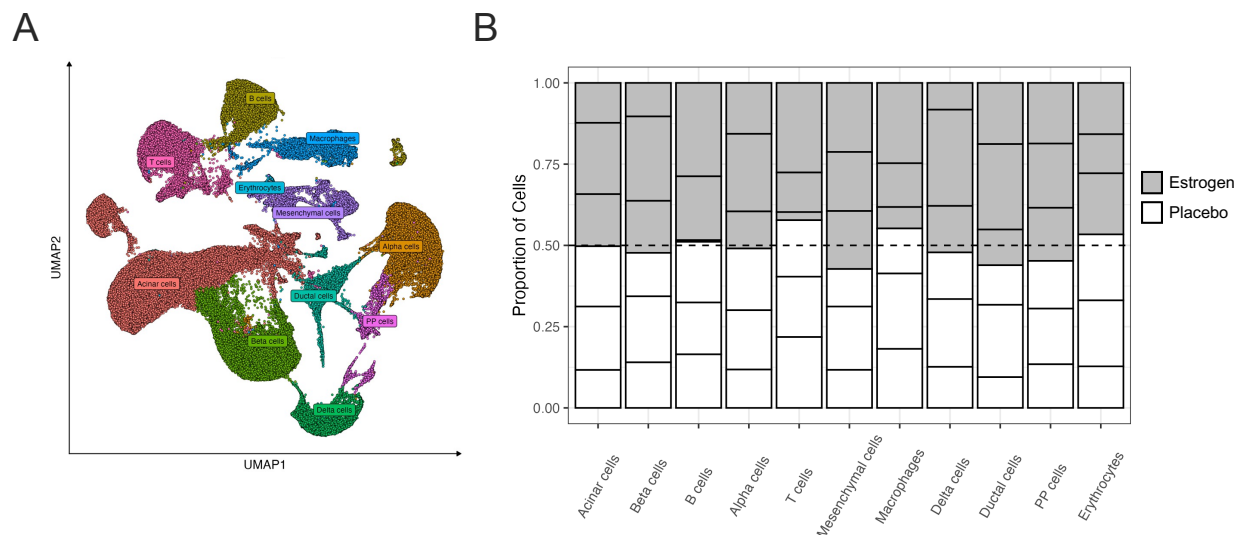


Figure 20: E2 treatment does not change the proportion of any major cell types present in the islet after 4 weeks by scRNAseq.

(A) UMAP projection of all cells detected by scRNAseq of islets isolated from female NOD mice treated with placebo or E2 pellet for 4 weeks. N=3 biological replicates per condition, each comprised of islets pooled from 2 mice. (B) Proportion of cells of each annotated cell type in the dataset contributed by each treatment group. Slices of each bar represent contributions of individual samples (N=3 per condition, Placebo and E2).

4.2.8 Estradiol treatment suppresses inflammatory signaling, β cell maturity genes, and antigen presentation pathways in the β cell.

I turned next to the transcriptional profiles of the endocrine cell types identified in the scRNAseq dataset: β cells, α cells, δ cells, and PP cells (**Figure 21A**). The overall frequency of each cell type was unchanged between treatment groups, save for a small increase in PP cells in the E2 samples (**Figure 21B**).

While relative numbers of β cells were unchanged, GSEA of the β cell population identified a wide swath of transcriptional differences between treatment groups, including suppression of Response to Interferon Beta (and Response to Type II Interferon), Antigen Processing and Presentation of Exogenous Antigens, and Lymphocyte Chemotaxis pathways in the E2-treated group (**Figure 21F**). Pathways *activated* in β cells by E2 treatment, on the other hand, primarily involved protein translation and mitochondrial ATP production, each of which are crucial to the maintenance of β cell biosynthetic functions like insulin production.

Among the IFN genes expressed at lower levels in β cells from E2-treated mice were known T1D risk gene *Ifih1* as well as genes like *Stat1*, *Irf1*, and *Isg15* that have been implicated in β cell death in T1D models^{203,271,272}. The β cell population was comprised of 5 clusters (**Figure 21C**). Intriguingly, the β cell cluster with the largest average shift in abundance with E2 treatment was defined by its expression of genes encoding antigen presentation machinery (e.g. MHC I genes *H2-K1*, *H2-D1*, *B2m*) and interferon response factors (e.g. *Irf1*, *Stat1*, *Stat2*) (**Figure 21C-D**). This cluster likely represents one that is highly inflamed and highly ‘visible’ to invading immune cells.

Evidence is mounting that inflammatory signaling by β cells themselves contributes to immune cell recruitment, activation, and cytotoxicity at the islet, and that quelling β cell

inflammation can protect against autoimmunity^{196,273}. Whether the suppression of type 1 IFN and antigen presentation is a direct effect of E2 signaling on the NOD β cells *in vivo* cannot be definitively concluded from the ‘snapshot’ provided here by scRNAseq. It is notable, however, that Interferon Gamma Response -a pathway which potentially induces MHC I expression- was suppressed by E2 in isolated human islets from both male and female donors treated directly with E2 in culture for only 18 hours (**Figure 15E-F**). It is also interesting to note that E2 treatment *in vivo* suppressed expression in β cells of several maturity genes, including *Slc2a2* (encoding Glut2), *Pdx1*, and *Nkx6-1* (**Figure 21E**). β cells with fewer ‘ β cell features’ have been observed to selectively survive the onslaught of autoimmunity in the NOD mouse; thus, E2 may also protect β cells from autoimmune destruction, in part, by reducing β cell features (e.g *Pdx1*) recognizable to islet autoreactive immune cells.

Overall, these data suggest that 4 weeks of E2 treatment decreased immunogenic β cell pathways including IFN signaling, chemokine production, and antigen presentation, as well as β maturity, all of which may contribute to the resistance to diabetes incidence observed in E2 treated mice in my and others’ studies.

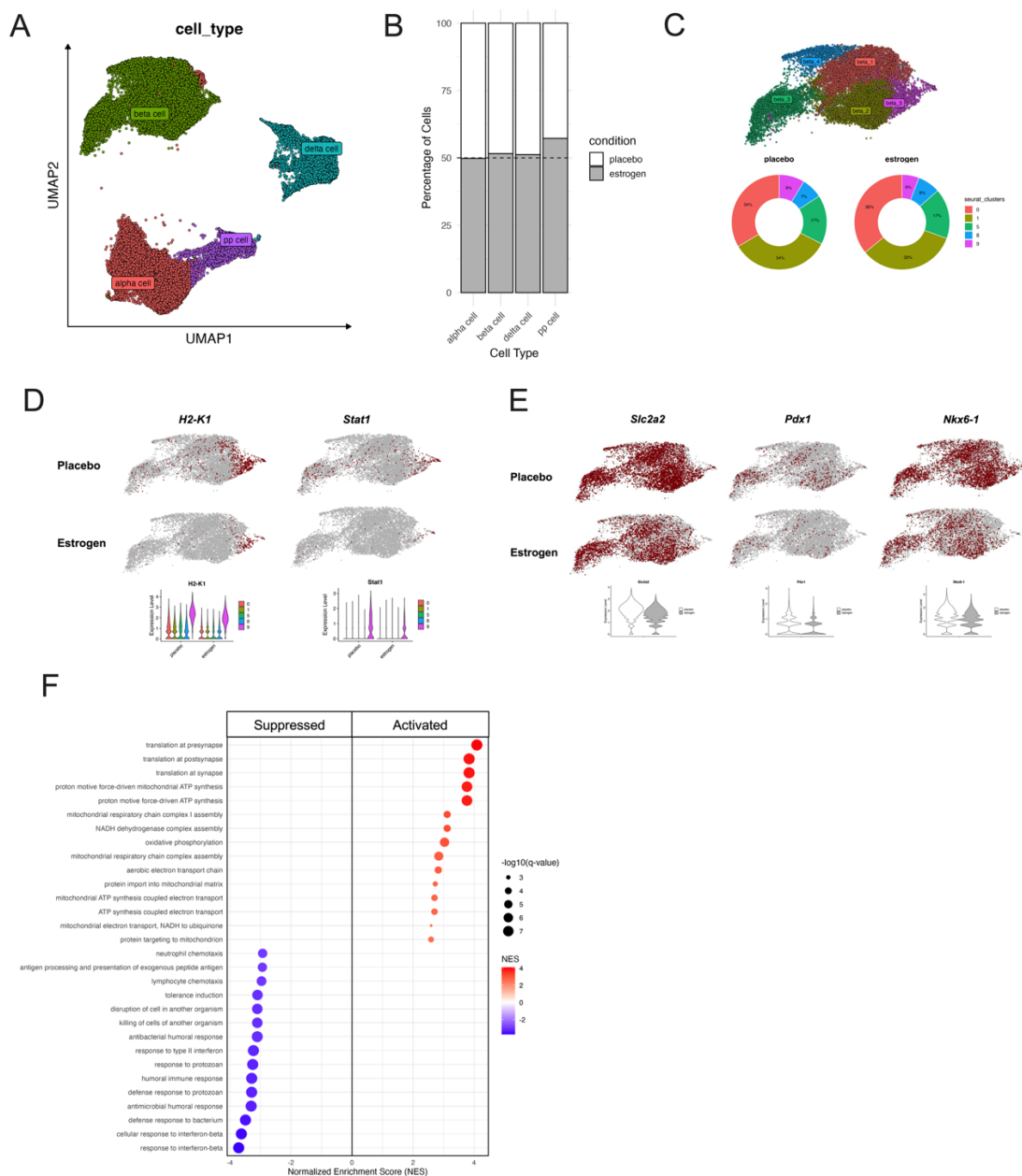


Figure 21: E2 suppresses inflammatory and antigen presentation pathways in β cells.

(A) UMAP of endocrine cell types present in the scRNAseq dataset. (B) Relative proportions of each endocrine cell type contributed by each treatment group. (C) UMAP representation of the 5 detected β cell clusters and donut plots displaying the percentage of β cells belonging to each cluster, by treatment group. (D) Feature plots of expression of *H2-K1* and *Stat1* among the β cell population in each treatment group. Below, violin plots representing expression of these genes across the 5 β cell clusters by treatment group. (E) Feature plots of expression of β cell identity genes among the β cell population, by treatment group. Below, violin plots representing expression of these genes across all β cells by treatment group. (F) GO-GSEA pathways activated (red) and suppressed (blue) by E2 treatment. Pathways with 15 highest and lowest NES displayed; all pathways have adjusted p-value<0.05.

4.2.9 Estradiol treatment suppresses proinflammatory, cytotoxicity pathways in the immune cell population.

Studies have demonstrated that reducing β cell inflammation (i.e. via β cell-specific KO of proinflammatory factors) can also generate less inflammatory phenotypes in islet immune cells^{196,273}. I next examined differential gene expression between treatment groups in the immune cell populations detected (**Figure 22A**). Relative proportions of macrophages, T cells, and B cells were similar between treatment groups; although of marginal statistical significance ($p=0.07$), dendritic cells were more abundant in the placebo group (**Figure 22B**). Notably, the dendritic cell population expressed markers of pDCs including *Siglech*, *Bst2*, *Tcf4*, and *Tlr7* (**Figure 22C**). pDCs are antigen presenting cells well-known as major producers of ‘key T1D trigger’ IFN- α , and this population is overrepresented in the peripheral blood of individuals with recent-onset T1D^{123,126,274}.

Among the genes most differentially expressed in immune cells between E2 and placebo were markers of cytotoxic effector cells, including cytolytic granzymes (*Gzma*, *Gzmb*) and perforin (*Prfl*); these were primarily expressed in a small cluster of *Cd8*-expressing T cells across treatment groups (**Figure 22D**). On the other hand, among the few genes more highly expressed in E2 immune cells than in placebo were *Cd163*, *Fol2r*, and *Mrc1*, each primarily expressed in macrophages and encoding markers of the anti-inflammatory M2-like phenotype (**Figure 22E**). GSEA of the immune compartment identified pathways enriched in placebo relative to E2 treated samples related to lymphocyte cytotoxicity, activation, and proliferation among many others, while pathways enriched in E2 treated samples again related mostly to mitochondrial ATP production and protein translation (**Figure 22F**).

Overall, these data demonstrate that E2 treatment suppressed proinflammatory signaling and cytotoxicity pathways in islet immune cells, enriched markers of anti-inflammatory M2-like macrophages, and reduced the abundance of DCs expressing markers of the pDC subtype, major producers of IFN- α which are increased in the circulation in new onset T1D²⁷⁴.

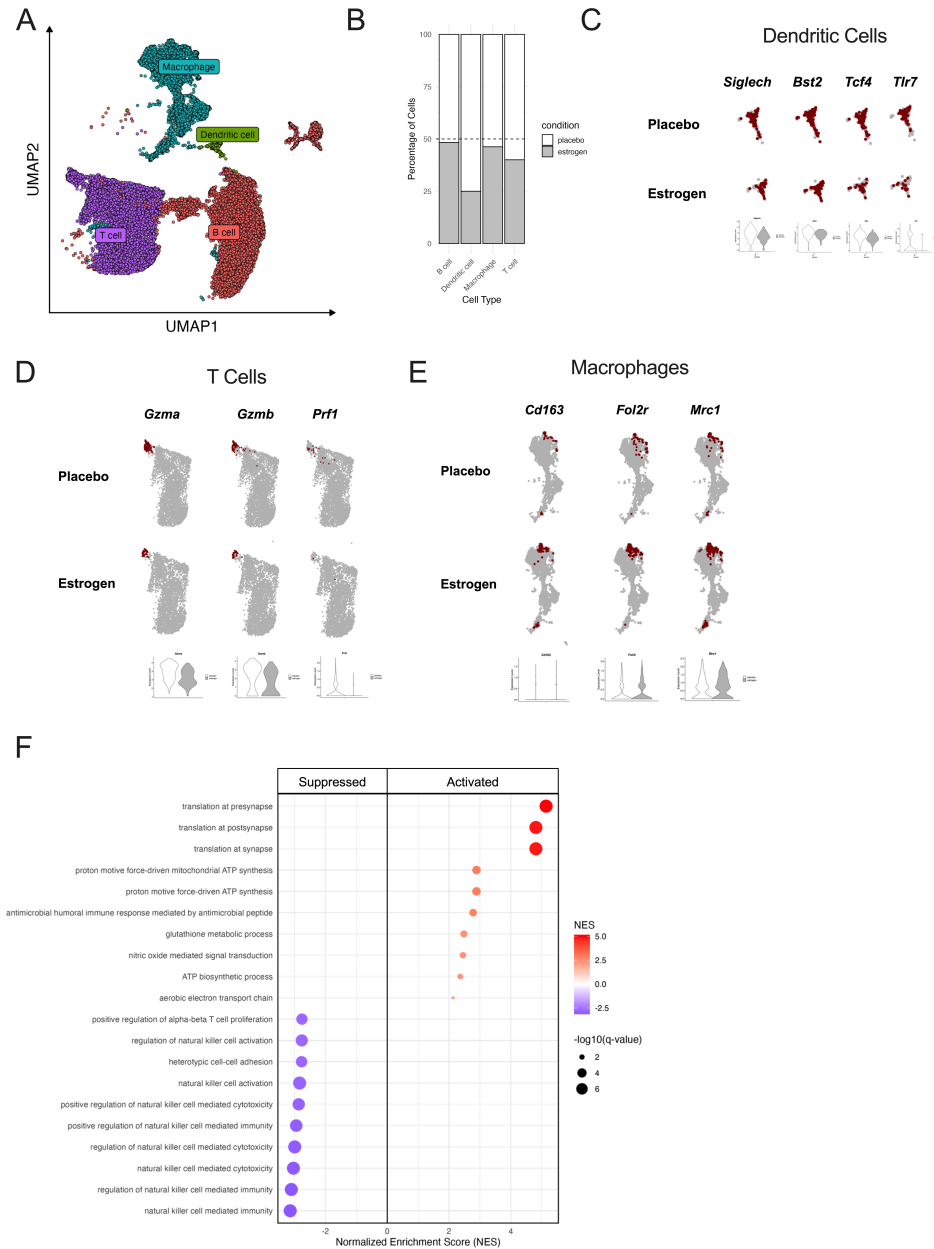


Figure 22: E2 suppresses pro-inflammatory, cytotoxic signaling in islet immune cells.

(A) UMAP of immune cell types. (B) Frequency of cell type by treatment group. (C) Feature plots of pDC marker gene expression among dendritic cells population. Below, violins show expression of these genes in DCs by treatment group. (D) Feature plots of cytolytic gene expression in the T cell population. Below, violins represent expression of these genes in T cell cluster 5 by treatment group. (E) Feature plots of M2 macrophage marker genes in the Macrophage population. Below, violins show expression of these genes in Macrophage cluster 3 by treatment group. (F) GO-GSEA pathways suppressed (blue) and activated (red) by E2 treatment. Pathways with 10 highest and lowest NES displayed; all pathways have adjusted p-value<0.05.

4.3 Discussion

In this chapter, I explored the influence of the sex hormone E2 on the islet transcriptome and proteome and on islet response to the early stages of autoimmune diabetes *in vivo*. I first demonstrated that the gene for ER α is upregulated in C57BL/6 and human islets in response to PIC (**Figure 12**), an effect absent in the islets of autoimmune diabetes-prone NOD islets (**Figure 17**), suggesting that E2 signaling plays a role in the islet inflammatory response. To better understand how E2 alone alters islet phenotype, I utilized acute E2 treatments of murine and human tissues *in vitro*. In mouse β cells and islets, I learned that E2 decreases expression of β cell maturity markers (i.e. *Pdx1* and *Ucn3*) while upregulating developmental pathway genes (i.e. *Hes1*) (**Figure 14**), findings which were mirrored in the β cells of NOD mice treated with E2 *in vivo* (**Figure 21**). Loss of β cell maturity markers is emblematic of β cell ‘dedifferentiation’, a phenomenon that has been studied as a mechanism of β cell failure during T2D^{20,21,275}. On the other hand, as discussed in the previous chapter, dedifferentiation may be a mechanism of protection via immune evasion available to β cells during autoimmunity^{211,212}. *Pdx1* also transactivates *Ovol2*, a repressor of the EMT²⁶⁰; thus, E2 exposure *in vivo* offers a potential mechanism for the enriched developmental signatures I observed in female islets (**Figure 7**; **Figure 9**).

Response to acute E2 treatment in human islets was highly sexually dimorphic at both the transcriptomic and proteomic levels, but with functional overlaps including transcriptional suppression of inflammatory pathways including Interferon Gamma Response (**Figure 15**; **Figure 16**). This divergence in response could possibly reflect differential chromatin accessibility between male and female islets, or signaling via different pools of estrogen receptors. At the proteomic level, for example, E2 modulated the “plasma membrane estrogen signaling” specifically in female islets (**Figure 16B**). Acute E2 responses were overall subtle

(e.g. with smaller effect sizes and more noise than PIC response, for example); the effect of *chronic* E2 exposure on the islet may be more likely to reflect the root of sex differences at the islet *in vivo*.

In the autoimmune diabetes-prone NOD model, chronic E2 administration via a slow-release pellet uniformly protected male and female mice from diabetes incidence (**Figure 18**). During the prediabetic period, this treatment did not expand β cell mass or entirely prevent insulinitis; E2 did, however, decrease the proportion of islets that were severely insulinitic (>50% area covered by immune infiltrates) (**Figure 19**). Differential expression analyses via scRNAseq revealed widespread transcriptomic changes in both the endocrine and immune cell compartments, however. In β cells, E2 suppressed type 1 and 2 IFN responses, lymphocyte chemotaxis, and antigen presentation pathways (**Figure 21**), a finding that mirrors both the relative suppression of IFN α response genes by GSEA in female human islets observed in Chapter 3 (**Figure 7**; **Figure 9**) and the suppression of IFN- γ Response by E2 treatment in islets from both sexes (**Figure 15**). IFN- α is well known to be an early contributor to T1D pathogenesis, with a type 1 IFN signature appearing in the serum before autoantibodies even develop^{126,127,194,230}. IFN α response genes are not only enriched in the β cells of AAB+ and T1D human donors (**Figure 11**), but are also a signature shared across target tissues of multiple common autoimmune disorders²⁷⁶. Signaling from both type 1 and type 2 IFNs are thought to contribute to β cell immunogenicity in part by increasing the production and presentation of neoantigens on MHC I complexes, which could then be engaged directly by the TCRS of CD8+ cytotoxic effectors^{230,277,278}.

The idea that E2 exposure could act directly on β cells to make them less ‘visible’ to cytotoxic immune cells via decreased antigen presentation is an intriguing one, particularly

alongside the notion that E2 may decrease β cell visibility via dedifferentiation or reactivation of developmental pathways (**Figure 14**; **Figure 21**). Fittingly, the phenotype I observe in the pancreata of E2 treated NOD mice mirrors the phenotype seen in β cell specific MHC-I null NOD mice; these mice develop insulinitis at similar rates to their wild-type counterparts, yet they do not develop diabetes²⁷⁹. Additionally, among the transcriptomic pathways modulated in E2-treated human islets of both sexes was Interferon Gamma Response (**Figure 15**), a potent inducer of MHC-I expression^{272,278,280}, suggesting that decreased antigen presentation could be a direct effect of E2 at the β cell.

In addition to its effects in β cells, E2 treatment also suppressed proinflammatory genes in NOD islet immune cells, including markers of cytotoxic effectors (e.g. *Gzma*, *Gzmb*, *Prfl*) and of IFN α -producing pDCs (e.g. *Tcf4*, *Siglech*, *Bst2*) (**Figure 22**). It is possible that this anti-inflammatory effect on immune cells occurs via direct E2 signaling at these cells, or it may be mediated through E2's anti-inflammatory effects in β cells. β cell-specific depletion of proinflammatory mediators such as *Alox15*, for example, has been shown to have ripple effects in islet immune cells, diminishing CD8⁺ IFN- γ ⁺ cytotoxic T-cells while increasing FoxP3⁺ regulatory T cells²⁷³.

Overall, the data presented in this chapter demonstrate that E2 protects the islet against the development of autoimmune diabetes, suppressing inflammatory signaling pathways implicated in T1D pathogenesis in both the β cell and islet immune cells while also suppressing antigen presentation pathways in the β cell. Ongoing work will investigate the directionality of this effect, parsing whether E2 signaling at the islet or in the adaptive immune cells alone are sufficient to protect from disease.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The “who, when, and why” of T1D onset remains mostly unresolved, even among individuals with risk HLA haplotypes or first-degree relatives with the disease. Meanwhile, T1D incidence has been on the rise for decades, a phenomenon thought to be driven by the interaction of genetic risk with an increasingly diabetogenic environment^{281–284}. Thus, it is crucial to understand how pancreatic islet response to environmental challenges like infection and inflammation might precipitate- or protect from- the development of islet autoimmunity. In this dissertation, I leveraged the observation that after the age of puberty, T1D incidence becomes more common in males^{144,145}, asking how sex influences islet response to the potential T1D trigger of inflammation.

In Chapter 3, I demonstrated across a discovery and a validation RNA sequencing dataset that male human islets display a larger proinflammatory transcriptomic response to PIC and are enriched for IFN α response genes- early predictors of islet autoimmunity in human T1D¹²⁶. In Chapter 4, I provided evidence that protective signaling by the sex hormone E2 suppresses IFN signaling in NOD islets *in vivo* and in human islets *in vitro*, suggesting that E2 may drive sexually dimorphic aspects of the islet response to PIC. By what mechanisms E2 or other aspects of sex precondition sexually dimorphic PIC responses, however, remains to be explored. Single cell ATAC sequencing of human islets suggests that chromatin accessibility differs between male and female β cells in nondiabetic donors²⁸⁵, and E2 has been noted to modify chromatin accessibility in other cell types^{286,287}. Future ATACseq studies comparing female β cells to male β cells treated with E2 could be used to test the hypothesis that the chronic exposure to E2 in female β cells decreases accessibility of inflammatory genes, thereby priming female islets for more tempered PIC responses.

Chronic E2 treatment *in vivo* uniformly protected from diabetes development in both male and female NOD mice. Examination of pancreata from E2 treated NOD mice showed no evidence of β cell mass expansion and, as in placebo controls, showed immune infiltration into the islets, albeit with decreased severity. Yet, inflammatory signaling- including type 1 IFN responses, leukocyte chemotaxis, and antigen presentation pathways- were significantly diminished in β cells from E2-treated mice. In addition to its suppression of inflammatory signaling, E2 also suppressed expression of major maturity markers in β cells including *Pdx1*, *Slc2a2*, and *Nkx6-1*, mirroring my *in vitro* findings in MIN6 cells. These changes could reflect β cell ‘dedifferentiation’, a phenomenon also noted to involve activation of EMT and other developmental pathways that I observed to be enriched in human islets from female donors. Thus, β cells in E2-treated mice are downregulating their expression of both antigen-presentation machinery and classical β cell antigens that might be presented on MHC-I; these may be two complementary approaches to reduce ‘visibility’ of β cells to cytotoxic effectors. Notably, cytotoxic and inflammatory pathways were also suppressed in islet immune cells in E2-treated mice. Whether anti-inflammatory effects at the endocrine and immune cells are independent or intertwined, however, is not yet clear.

Studies have demonstrated that β cell specific depletion of proinflammatory factors (e.g. *Alox15*, *Tet2*) on the NOD background not only protects from β cell death and diabetes development, but also increases expression of immune inhibitory markers (e.g. PD-L1) and decreases markers of cytotoxic effectors (e.g. *Gzmm*, *Gzmk*) on invading immune cells. To determine whether anti-inflammatory effects of E2 I observed in the islet could, in turn, produce the anti-inflammatory effects observed in islet immune cells, I will perform a set of *in vitro* and *in vivo* experiments. *In vitro*, I will co-culture islets pretreated with E2 or vehicle with naïve T

cells, and the resulting T cell activation states (i.e. Th1, Th2, Treg) will be examined by flow cytometry and supernatant ELISA for proinflammatory cytokines. *In vivo*, I will adoptively transfer diabetogenic, islet-targeted T cells from the BDC2.5-NOD mouse line^{113,288,289} into NOD.SCID recipients pretreated with a placebo or E2 pellet for 4 weeks. In these experiments, the recipients lack adaptive immune cells of their own, and the T cell donors will not be treated with E2. Likewise, the reverse experiment can be performed, with T cell donors receiving E2 while the recipients (and thus, the islets) are not exposed to exogenous E2. Thus, I will determine whether E2 treatment including the islets and excluding adaptive immune cells- or vice versa- is sufficient to protect from autoimmune diabetes.

While NOD mice are a strong model of autoimmune diabetes pathophysiology with many parallels to human T1D, it is essential to draw connections back to human tissues to ensure translational relevance of NOD findings. Human islet responses to acute E2 treatment *in vitro* mirrored some of the protective effects observed in NOD islets with chronic E2 treatment- namely, decreases in IFN and cell death pathways. As with PIC, however, these responses were highly sexually dimorphic. Contrary to popular thought, E2 plays well-established roles in both male and female physiology, from reproduction to metabolism^{290,291}. Islet estrogen receptor expression and aromatase activity- converting testosterone to E2 locally- have been demonstrated in both sexes²⁹²⁻²⁹⁴. Whether receptor expression levels vary between sexes at the islet has not been reported. Genomic signaling via ERs also relies on a host of co-activators and co-repressors whose expression and activity have not been characterized between male and female islets. In addition to their function as nuclear receptors, ERs can also associate with the plasma membrane and transduce rapid non-genomic signaling in response to ligand-binding, representing yet another potential point of divergence in islet E2 signaling between sexes.

Interestingly, proteins related to “plasma membrane estrogen signaling” were modulated specifically in female human islets by E2 treatment. Future experiments assessing estrogen receptor subtype expression and activity between male and female islets, taking advantage of selective agonists (i.e. PPT for ER α , G1 for GPER1) or single receptor knockdown, will serve to clarify the details of islet E2 action between sexes.

It is also notable that while male and female NOD mice were both protected from diabetes development by E2 treatment, it is unclear whether they were protected via the same mechanisms, as we were only able to perform scRNAseq on islets from *female* placebo and E2-treated mice. I did, however, collect islets from male NOD mice treated with placebo or E2 pellets. Future analysis of these chronically E2-treated tissues via bulk RNA sequencing or qRT-PCR could complement our analyses in acutely E2-treated human islets.

The work presented in this dissertation adds to growing evidence of favorable actions of E2 on β cell function and survival. How this protection might translated into therapeutic benefit in T1D is interesting to consider. My study and others^{162,269} demonstrate preventive action against diabetes development, addressing a fundamental gap in our current toolkit for individuals at high risk of T1D. While immunosuppression via teplizumab treatment offers some delay of T1D onset, perhaps E2 could serve as an adjunctive therapy targeting β cell function and survival. E2, to my knowledge, has not been tested therapeutically in mice or humans who have already developed autoimmune diabetes; whether E2 may be able to reverse islet inflammation or preserve β cell mass should be investigated. Given potential undesirable effects of systemic E2 administration on sex characteristics or sex hormone responsive organs (e.g. uterine hypertrophy), other investigators have developed a dual agonist fusing E2 to GLP-1, compounding the β cell benefits of the monoagonists while also creating more cell targeting

specificity^{295,296}. Whether the GLP-1 E2 dual agonist would provide additional protections in NOD or human autoimmune diabetes, either in the diabetic or prediabetic stage, are clear avenues for future investigation.

In conclusion, I have demonstrated that human islets from male and female donors display sexually dimorphic responses to PIC, a potential trigger for T1D development, with male islets showing enrichment in pathways like IFN α known to drive T1D pathophysiology. Sex hormone E2 induces changes in mouse and human islets that mirror the sexual dimorphism observed in PIC response, and E2 treatment protected male and female NOD mice from autoimmune diabetes *in vivo*. These findings indicate that chronic exposure of female islets to E2 may indeed lend protection from diabetogenic insults and contribute to the male predominance observed in human T1D.

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