

Progress Report

The Clinical Problem

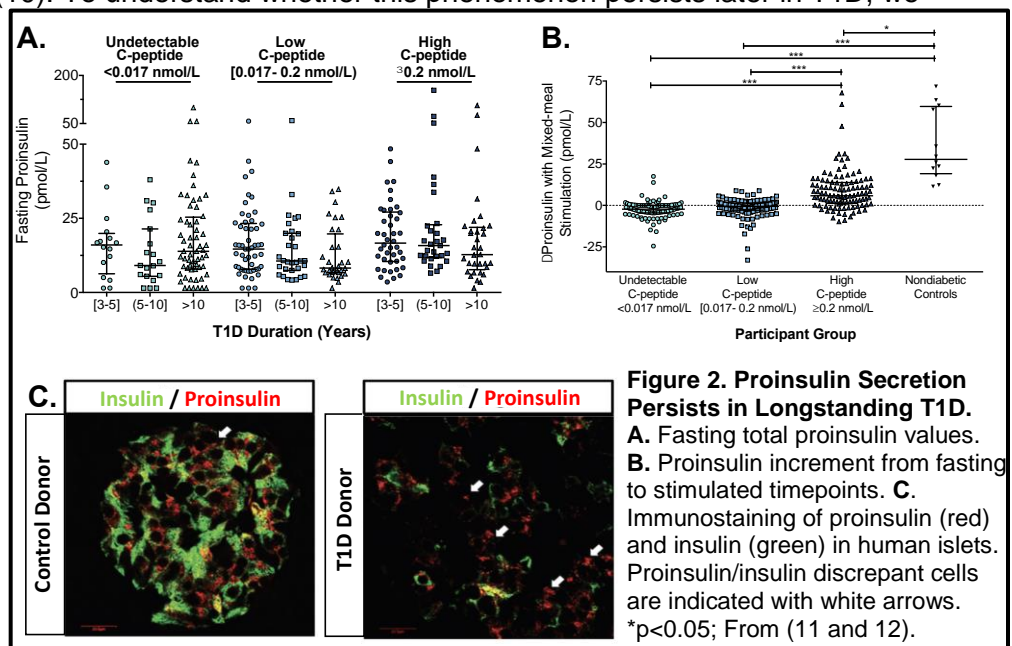
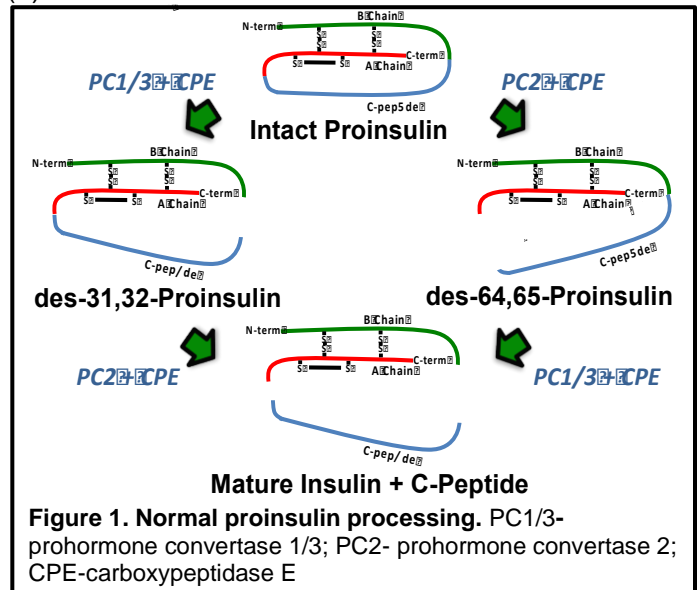
The global incidence of Type 1 Diabetes (T1D), a disorder commonly beginning in childhood, is increasing at nearly 3% per year (1, 2). Whereas immunomodulatory therapies have shown some efficacy to attenuate T1D, their long-term effectiveness has been limited (3). These failures are partly related to irreparable damage to the functional β cell pool that has accumulated by the time of clinical presentation. Emerging data also suggest β cells themselves may not be innocent bystanders in T1D development. Rather, metabolic stress pathways intrinsic to the β cell, which may be initially activated to increase cell survival and insulin production, can ultimately hasten the progression to apoptosis or enhance β cell responsiveness to immune infiltration and destruction (4). These findings suggest that limited efficacy of immunotherapies may also reflect inadequate treatment of T1D-related β cell dysfunction, and highlight two **critical needs** in the field: 1) identification of noninvasive biomarkers that would better enable longitudinal monitoring of β cell health and 2) an enhanced molecular understanding of the β cell's contribution to T1D pathophysiology. Such strategies would improve therapeutic outcomes by allowing T1D detection before the onset of irremediable β cell dysfunction and death, and by paving the way for novel β cell targeted therapies (5).

Altered Proinsulin Processing in T1D

As a secretory endocrine cell, the β cell requires robust and efficient processing of proteins, including insulin. Under normal conditions, proinsulin disulfide bond formation and terminal protein folding occur in the endoplasmic reticulum (ER) and Golgi, and intact proinsulin is eventually cleaved into mature insulin and C-peptide by processing enzymes within secretory granules (6). Under conditions that impair β cell health, such as inflammatory or oxidative stress, insulin demand may exceed the ability of the β cell ER to process newly translated proteins. When unchecked, this leads to ER stress signaling and β cell death (7). A hallmark of β cell dysfunction is the accumulation and secretion of inadequately processed proinsulin (7). Thus, β cell intrinsic stress may be detected noninvasively by measurement of increased circulating proinsulin relative to circulating mature insulin or C-peptide (8). We have previously demonstrated that, in at-risk islet autoantibody positive relatives, increased serum proinsulin relative to C-peptide is independently associated with T1D progression (9). At the time of T1D onset, circulating proinsulin relative to C-peptide is ~3-fold increased compared to controls (10). To understand whether this phenomenon persists later in T1D, we

analyzed fasting and stimulated serum samples from 341 individuals with longstanding T1D (> 3 years) that were previously tested for stimulated serum C-peptide levels (**Figure 2A**) (11). Strikingly, fasting total proinsulin levels were similar among participants with undetectable, low, or high residual C-peptide levels (defined as <0.017 nmol/L, ≥ 0.017 -0.2 nmol/L, or ≥ 0.2 nmol/L). Specifically, we were able to detect fasting proinsulin in 89.9% percent of subjects with undetectable stimulated C-peptide.

However, proinsulin only increased with mixed meal stimulation among



individuals with highly functioning residual β cells, reflected by high stimulated C-peptide values (**Figure 2B**). These findings suggest that in poorly functioning β cells, constitutive proinsulin release may occur through alternative pathways, distinct from glucose-stimulated secretion of insulin granules. Proinsulin and insulin immunostaining in human islets revealed that multiple T1D donor islets exhibited numerous cells with a proinsulin enriched, insulin poor phenotype (Example in **Figure 2C**) (12). In aggregate, this work suggests that dysfunctional β cell proinsulin processing and release contribute to insulin deficiency in all stages of T1D.

Beta Cell Exosomes in Health and Disease. Extracellular Vesicles (EVs) are membrane bound nanoparticles housing molecular cargo (13). EVs can be transferred to or interact with other cells as a means of cell:cell communication (Fig. 6A) (13-27). Our data suggest that the most abundant islet and β cell EVs are exosomes: small EVs formed from exocytosis of multivesicular endosomes (MVEs) (Fig. 6B-E). Findings from our lab and others suggest that β cell exosomes serve as paracrine effectors in the islet microenvironment in health and disease (5, 16, 18, 28-35). For example, cytokine treatment of β cells to induce inflammatory stress induces physiologic changes in exosome cargo that impacts surrounding islet cells (28, 31-33). Specifically, our group has demonstrated that islet inflammatory stress increases beta cell exosome miR-21 cargo (21). Consistent with this, EV miR-21 progressively increases in the sera of nonobese diabetic mice before the onset of diabetes, and is increased in serum from children with T1D compared to nondiabetic controls (21). Other groups have shown that islet exosomes activate antigen presenting cells, and stimulate endothelial cell angiogenesis (28-30). Exosomes from cytokine-treated islets display enhanced immunostimulatory properties (28). Conversely, T lymphocyte exosomes can transfer miRNAs to β cells that induce apoptosis and chemokine signaling (36).

Diverse molecular mechanisms have been identified as regulating exosome biogenesis and cargo loading in non-islet cells, including: 1) Ceramide dependent MVE vesicle formation and release of ceramide-enriched exosome subpopulations (37-41); 2) Endosomal sorting complex required for transport (ESCRT)-dependent MVE formation and release (42); 3) Syndecan-syntenin-ALG-2-interacting protein X complex induction of MVE vesicle intraluminal budding (43); 4) Preferential localization of miRNAs bound to RNA silencing complexes (miRISC complexes) at MVE biogenesis sites (44); 5) Rab GTPase mediated MVE fusion with the plasma membrane (45); and 6) miRNA association with sumoylated RNA binding proteins (13, 46-50). However, mechanisms and physiologic impacts of changes in β cell exosome cargo remain poorly defined.

Hypothesis: Intriguingly, islet exosomes can contain proinsulin cargo (28). However, the mechanisms underlying these changes in proinsulin cargo remain untested. Based on our prior work, our **central hypothesis** is that ***β cells under proinflammatory stress release increased extracellular proinsulin via increased packaging of proinsulin cargo into β cell exosomes.***

Progress over the period of the ISPAD Fellowship

Experimental Progress:

Over the period of this award, most of our progress has occurred in **Specific Aim 2**, in which we proposed to define physiologic relevance of changes in β cell exosome proinsulin content under conditions of inflammatory stress by testing the hypothesis that β cells release proinsulin in exosomes as a compensatory response to dispose of accumulating intracellular proinsulin during cytokine-induced ER stress.

First, we treated INS-1 clonal β cells with 24 hrs of 5ng/mL IL-1 β . Exosomes were isolated using size exclusion chromatography (Izon SEC columns) of exosome-depleted media. Exosome proinsulin was measured using a total proinsulin ELISA (Mercodia) and normalized to total cellular proinsulin. As shown in **Figure 3A**, we observed increased proinsulin in exosomes derived from cytokine-treated cells. Nanoparticle tracking analysis

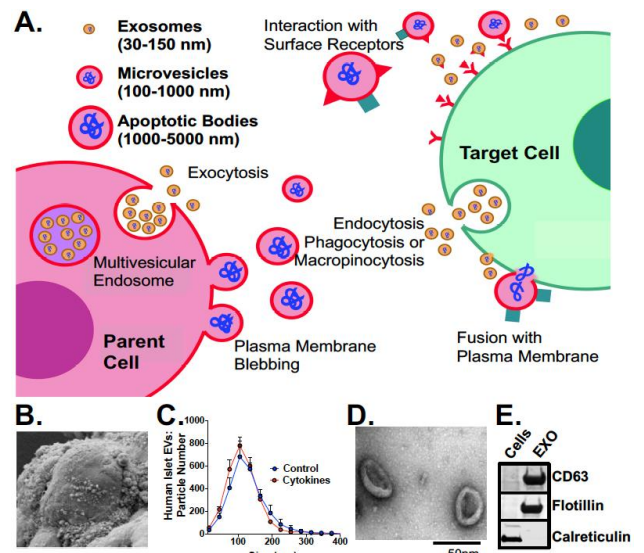


Figure 3. EVs as potential paracrine effectors in islet health.
A. EV categories, origins, and interactions with target cells. **B.** Scanning electron micrograph (EM) of EVs on the extracellular plasma membrane surface of a human islet cell. **C.** Nanoparticle tracking analysis on control and cytokine treated human islet EVs. **D.** Transmission EM of human islet exosomes **E.** Immunoblot on human islet cells and exosomes for exosome markers (CD63 and

(NTA, **Figure 3B-C**) to quantify total extracellular vesicles (EVs) pointed away from an increase in total exosome release or size distribution after cytokine treatment, suggesting that our findings were due to changes in exosome cargo rather than shifts in global exosome counts.

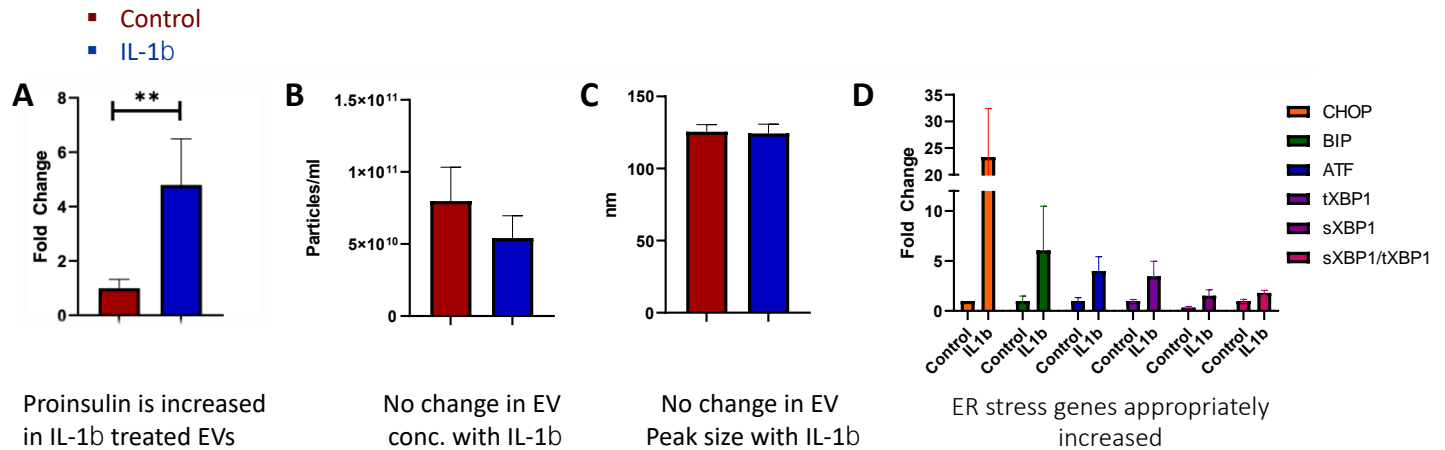


Figure 4. Exosomal proinsulin is increased in INS-1 cells after treatment with IL-1 β . A. Quantification of exosomal proinsulin (ELISA) B-C. Nanoparticle tracking analysis showing total EV count and peak size. D. qRT-PCR was performed to verify upregulation of genes associated with ER stress.

Next, we treated INS-1 clonal β cells with 24 hrs of tunicamycin to induce ER stress directly. Exosomes were isolated and exosome proinsulin was measured and normalized as above. Here, we observed that direct induction of ER stress was associated with an even more pronounced increased in exosomal proinsulin content.

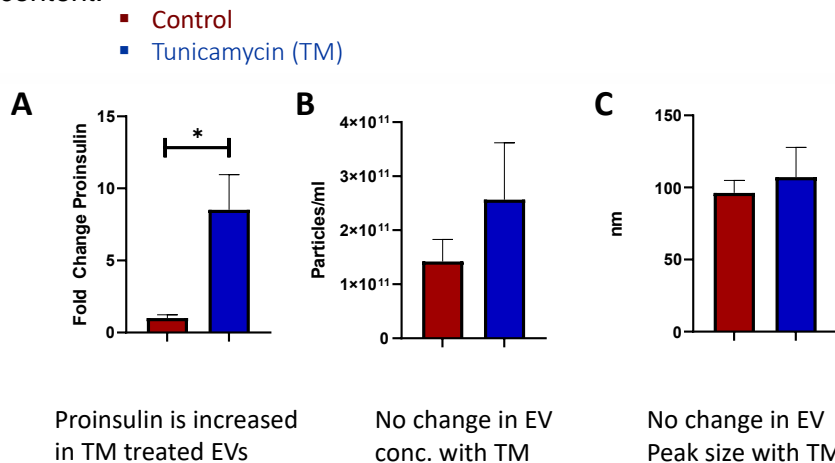


Figure 5. Exosomal proinsulin is increased in INS-1 cells after treatment with tunicamycin to directly induce ER Stress. A. Quantification of exosomal proinsulin (ELISA) B-C. Nanoparticle tracking analysis showing total EV count and peak size.

We also performed analysis of exosomes isolated from islets from C57BL6/J mice treated with or without cytokines and NOD mice. Exosomes were isolated with size exclusion chromatography. However, here, total exosome counts were very small with significant variability, making interpretation of results challenging.

For testing in human clinical samples, although we have identified samples for testing, given variability in our preclinical data we have chosen to wait until we have optimized isolation methods to proceed with testing on human samples.

Scientific Interpretation and Next Steps: Our data suggest that β cell exosomal proinsulin is increased with cytokine treatment and further increased by direct induction of ER stress, suggesting that cytokine-induced ER stress may be an important mechanistic contributor. For future experiments with primary cells, we will combine islets multiple mice as one replicate to generate larger numbers of exosomes. We also plan to test different methods of exosome isolation to determine if these yield differences in variability of proinsulin content. Finally, we plan to compare our findings using intact vs. total proinsulin assays to determine if different proinsulin species are preferentially altered in β cell exosomes. Our final step will be analysis in human clinical samples.

Presentations and Publications: These data were presented as an oral abstract at the 2021 Pediatric Endocrine Society meeting. Once finalized, we will plan to submit as an original manuscript to a diabetes journal such as *Diabetologia* or *Diabetes*.

Future Funding Applications: These data were also used as preliminary data for an R01 application to NIDDK based on defining mechanistic etiologies of altered proinsulin secretion in T1D.

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