Immunomodulatory responses in the pancreas in health and disease

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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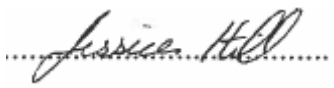

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Abstract

Loss of β-cell mass is a key mechanism leading to the development of Type 1 diabetes (T1D). However, this process can be gradual with many patients retaining significant portions of their normal β-cell mass during the first 1-3 years from clinical diagnosis. The mechanisms underlying this progressive loss of β-cell mass are not fully elucidated but the 'crosstalk' between the immune system and β-cell has increasingly gained focus. The findings in this thesis challenge the notion that the islets of Langerhans are defenceless against an immune attack, and demonstrate that expression of immune checkpoint proteins PD-L1 and HLA-E are both present in individuals with T1D, and absent from donors without T1D. The expression of these proteins is associated with upregulation of interferon-stimulated genes, which are known to be active in early T1D pathogenesis. In addition, this thesis demonstrates that PD-L1 can be expressed on the surface of extracellular vesicles released from β-cell-like cells (EndoC-βH1 cells), potentially indicating a mechanism for β-cells to enhance binding of PD-L1 to PD-1 on cytotoxic T cells to negatively regulate the immune attack. The positive correlation between PD-L1 and levels of insulitis in T1D individuals indicates that PD-L1 on extracellular vesicles, in the future, may be able to act as biomarkers of disease progression.

This thesis also explores the immunomodulatory responses in the pancreas when challenged by Type 3cDM diabetes associated with pancreatic ductal adenocarcinoma (PDAC). Findings indicate that individuals with diabetes and PDAC do not differ in immunologic profile compared to individuals with no diabetes, and therefore diabetes is not a marker for altering clinical management of PDAC.

Finally, this thesis explored the epigenetic characterisation of a persistent coxsackie viral infection in pancreatic cells to demonstrate that no immunomodulatory mechanisms are altered with the establishment of persistence, but cells undergo changes in pathways for virus-host interactions, mitochondrial organisation, cellular protein catabolism, DNA damage responses and cellular responses to cadmium ions.

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Abbreviations

3’ UTR or 5’ UTR	3’ or 5’ untranslated region
AAb	Autoantibody
ACh	Acetylcholine
ACTB	Actin, beta
ADST	Antibody diluting solution with Triton-X
ALIX	Programmed cell death 6-interacting protein also known as (PDCD6IP)
ANXA2	Annexin A2
ANXA5	Annexin 5
AP	Alkaline Phosphatase
APP	Amyloid precursor protein
ATCC	American Type Culture collection
ATP	Adenosine triphosphate
ARF6	ADP Ribosylation Factor 6
BB rat	Diabetes prone bio-breeding rat
BHLHA15	Basic Helix-Loop-Helix Family Member A15 (also known as MIST1)
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate

CAR	Coxsackie and adenovirus receptor
CBV	Coxsackie B virus or Coxsackie virus B
CD	Cluster of differentiation
CELA3	Chymotrypsin Like Elastase 3A
CFTR	Cystic fibrosis transmembrane conductance regulator
Cryo-EM	Cryo-electron microscopy
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	C-X-C Motif Chemokine Receptor 4
DAB	3,3′-Diaminobenzidine
DAISY	Diabetes and Autoimmunity Study in the Young
DCDC2	Doublecortin domain-containing protein 2
DIC	Differential interface contrast microscope
DIPP	Type 1 Diabetes Prediction and Prevention
DiViD	Diabetes Virus Detection Study
EADB	Exeter Archival Diabetes Biobank

ECACC	European Collection of Authenticated Cell Cultures
eIF2α	Eukaryotic Translation Initiation Factor 2A
ELISA	Enzyme-linked Immunosorbent Assay
ENO1	Enolase 1, (alpha)
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EV	Extracellular vesicle
Exo	Exosome
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
LFA1	Integrin Subunit Alpha L
FFPE	Formalin-fixed Paraffin embedded
FOXA2	Forkhead box protein A2
FRT	Flp Recombination Target
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GABA	Gamma aminobutyric acid
GADD65	Glutamic acid decarboxylase 2

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma-interferon activation sites
GCG	Glucagon
GIP	Gastric inhibitory polypeptide
GLP1	Glucagon-like peptide-1
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GPCR	G-Protein coupled receptor
GSIS	Glucose stimulated insulin secretion
GWAS	Genome-wide association studies
HbA1c	Glycated haemoglobin
HES1	Hes family bHLH transcription factor 1
hGH	Human growth hormone
HIER	Heat induced epitope retrieval
HLA	Human Leukocyte antigen
HNF4G	Hepatocyte nuclear factor 4 gamma
HNF4A	Hepatocyte nuclear factor-4 alpha
HNF1A	Hepatocyte nuclear factor-1 alpha
HNF1B	Hepatocyte nuclear factor-1 beta
HRP	Horse radish peroxidase
HSPA8	Heat Shock Protein 8

HSPG	Heparan Sulfate Proteoglycan 2
HSP90AA1	Heat shock protein 90, alpha (cytosolic), class A member 1
IA-2	Islet antigen 2 (Protein tyrosine phosphatase like protein)
IAA	Insulin autoantibody
ICI	Insulin containing islet
ICLAC	International cell line authentication committee
IDI	Insulin deficient islet
IFIH1	Interferon Induced With Helicase C Domain 1
IFN	Interferon
IgG	Immunoglobulin G
IL-15	Interleukin 15
IL-15Rα	Interleukin 15 receptor α
ILV	Intraluminal vesicles
INS	Insulin
IRF1	Interferon regulatory factor 1
ISL1	Islet1 transcription factor, LIM/homeodomain
ISG15	Interferon simulated gene 15
ISRE	Interferon-stimulated response element
ITGA5	Integrin Subunit Alpha 5
JAK	Janus kinase

JDRF	Juvenile Diabetes Research Foundation
KRB	Krebs ringer buffer
KRT19	Keratin 19
LADA	Latent autoimmune diabetes of adulthood
LCM	Laser capture microdissected
MAFA	musculoaponeurotic fibrosarcoma oncogene family, A
MCS	Multiple cloning site
MDA5	Melanoma differentiation-associated protein 5
MODY	Maturity onset diabetes of the young
MVB	Multivesicular body
NADPH	Nicotinamide adenine dinucleotide phosphate
NEUROD1	Neurogenic differentiation 1
NKX2-2	NK2 Homeobox 2
NGS	Normal goat serum
NOD mouse	Non-obese diabetic
nPOD	Network for Pancreatic Organ Donors with Diabetes
NR5A2	Nuclear Receptor Subfamily 5 Group A Member 2

PAX4	Paired box gene 4
PAX6	Paired box gene 6
PBS	Phosphate buffered saline
PC1/3	Prohormone convertase 1/3
PDAC	Pancreatic adenocarcinoma
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PDX1	Pancreatic and duodenal homeobox 1
PFA	Paraformaldehyde
PI	Propidium iodide
PKA	Protein kinase A
PKM	Pyruvate kinase, muscle
PKR	Protein kinase R
PMEL	Premelanosome protein
Poly:IC	polyinosinic:polycytidylic acid
PP1	Protein phosphatase 1
PPP1R1A	Protein phosphatase 1, regulatory, inhibitory, subunit 1A
PRSS1	Serine Protease 1
PRSS3	Serine Protease 3
PVDF	Polyvinylidene difluoride
RBPJL	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region Like

RCF	Relative Centrifugal Force or “g”
RRP	Readily releasable pool
SCTR	Human secretin receptor
SD	Standard Deviation
SNP	Single nucleotide polymorphism
SSC	Side scatter
SST	Somatostatin
ST8SIA1	α-N-acetylneuraminide α-2,8-sialyltransferase
STAT1	Signal transducer and activator of transcription 1
STAT1	Signal transducer and activator of transcription 2
STR	Short tandem repeat
T1D	Type 1 diabetes
T1 IFN	Type 1 interferon
T2D	Type 2 diabetes
TBS	Tris-buffered saline
TCA	Tricarboxylic acid cycle
TDP43	TAR DNA Binding Protein
TEDDY	Environmental Determinants of Diabetes in the Young
Tet	Tetracycline
TFF2	Trefoil Factor 2

TGFBR2	Transforming Growth Factor Beta Receptor 2
TP53	Tumour protein p53
TP63ΔN	Tumor protein p63, N-terminally truncated
TRIGR	Trial to Reduce Insulin-Dependent Diabetes Mellitus in the genetically at-risk
TSPAN7	Tetraspanin protein family member 7
TYK2	Non-receptor tyrosine-protein kinase
UPR	Unfolded protein response
VDCC	Voltage-dependent calcium channels
VP1	Viral protein 1
WNT	Wingless and Int-1
WT	Wild-type
ZnT8	Zinc transporter 8

Chapter 1

1 Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus is a collection of diseases characterised by dysregulation of glucose metabolism due to functional impairments in the secretion and/or peripheral actions of pancreatic hormones (especially insulin and glucagon) leading to hyperglycaemia. Individuals experiencing chronic hyperglycaemia can develop a number of secondary complications including nephropathy, peripheral neuropathy, autonomic neuropathy, cardiovascular disease and retinopathy. Without appropriate intervention, some individuals are at risk of amputation, blindness and kidney failure, therefore early diagnosis and effective treatment of Diabetes Mellitus is critical. Many must commit to life-long daily injections of exogenous insulin in order to survive, but the precise mode of diabetes management is dependent on the class of disease. The two main types of diabetes are described quite simply as Type 1 (T1D) and 2 (T2D). There are similarities in the presentation of symptoms between T1D and T2D, which can sometimes lead to misdiagnosis and additional problems as the two types of diabetes often demand different courses of management (Thomas and Philipson, 2015).

Less common forms of diabetes include gestational diabetes mellitus, maturity onset diabetes of the young (MODY), neonatal diabetes and an intriguing form of diabetes that can occur as a consequence of pancreatic ductal adenocarcinoma (PDAC), also referred to as Type 3c diabetes (T3cDM).

1.1.1 Monogenic Diabetes

Monogenic forms of diabetes include maturity onset diabetes of the young (MODY) and neonatal diabetes.

1.1.1.2 Maturity onset diabetes of the young (MODY)

MODY is a very specific form of diabetes that occurs as a result of specific genetic mutations, rather than combined influences of environmental conditions and genetic predisposition, as occurs in other types of diabetes. This also means that most individuals with MODY are diagnosed before they reach the age of twenty-five years (https://www.diabetes.org.uk/diabetes-the-basics/other-types-of-diabetes/mody; accessed 18/05/21). MODY is described as a rare type of diabetes and accounts for 1-2% of all people with diabetes. The four most common mutations seen in MODY are present within genes encoding HNF1-α, HNF4-α, HNF1-*β* and Glucokinase but several other mutations can also occur in genes such as *INS, KCNJ11* and *ABCC8* (Urakami, 2019). HNF1-α mutations account for around 70% of MODY cases and they alter the function of this transcription factor which is involved in the embryonic development of β-cells and also regulates insulin expression, and the glucose transporter protein, GLUT2 (McDonald and Ellard, 2013).

1.1.1.3 Neonatal diabetes

Neonatal diabetes is a form of diabetes attributed to patients diagnosed within the first six months of their life and results from the mutation of a single gene. This genetic mutation can either result from familial inheritance or arise via a sporadic *de novo* mutation. Mutations that lead to the development of neonatal diabetes most often impair β-cell function, though some have been found to impact insulin resistance (Musso et al., 2004, Hattersley et al., 2009). Genes most often affected include *INS, KCNJ11, ABCC8, EIF2AK3, FOXP3* and *GCK* (Hattersley et al., 2009, Barbetti and D'Annunzio, 2018). Neonatal diabetes is divided into permanent neonatal diabetes mellitus (PNDM) and transient neonatal diabetes mellitus (TNDM). Mutations in *KCNJ11* and *ACCB8* are common in PNDM and these encode the Kir6.2 and the SUR1 subunits,

respectively, of the ATP-sensitive K+ channel in pancreatic islets (Gloyn et al., 2004). TNDM is typically diagnosed in the first week of life with hyperglycemia and negative urinary ketones. The insulin requirement is lower in children with TNDM compared with PNDM (Metz et al., 2002), and decreases with time, leading to insulin independence by a median age of 12 weeks (Temple et al., 2000). However, the majority of patients have a relapse of their diabetes in later life. As well as mutations in *KCNJ11* and *ACCB8* , mutations in the *ZFP57* gene encoding a zinc-finger transcription factor linked to DNA hypomethylation of multiple imprinted loci is reported to be associated with TNDM in several cases (Mackay et al., 2008).

1.2 Type 2 Diabetes

Type 2 diabetes (T2D) is the most common form of diabetes accounting for almost 90% of all cases, and this number is rising steeply across the globe (https://www.diabetes.org.uk/type-2-diabetes, accessed 16/05/2021). T2D occurs as a result of genetic and environmental risk factors, the most recognisable of which being obesity (Fletcher et al., 2002). There is no isolated single genetic mutation associated with T2D and GWAS studies having identified over 143 risk variants (Spracklen et al., 2020, Noordam et al., 2021, Flannick et al., 2019)). T2D occurs when β-cells alter their function and peripheral tissues transition into a state of insulin resistance, which puts pressure on the β-cells to produce more insulin leading to their eventual failure to produce sufficient hormone.

Treatment of T2D can include adherence to a highly caloric restricted diet (Lean et al., 2018, Lean et al., 2019) but pharmaceutical intervention is often required. The first line of treatment is often metformin together with lifestyle intervention,

but if this is unsuccessful than second-line drugs include sulfonylureas and glitazones. Alternatively basal insulin can be prescribed with metformin (Fonseca and Kulkarni, 2008).

1.3 Pancreatic ductal adenocarcinoma associated diabetes (T3cDM)

An association between pancreatic ductal adenocarcinoma (PDAC) and diabetes has long been observed (Song et al., 2015, Bosetti et al., 2014), which is of clinical interest to physicians due to the possibility of finding a diabetes signature associated with PDAC that could help predict early stages of tumour growth (Pannala et al., 2009, Pelaez-Luna et al., 2007, Illés et al., 2016, Ben et al., 2011). The association between long-duration diabetes and PDAC is well documented but the pathogenesis of recent-onset diabetes associated with PDAC, also known as Pancreatogenic (Type 3c) Diabetes or T3cDM, is less well understood (Andersen et al., 2013). Epidemiological studies have shown that diabetes is a risk factor for PDAC (Ben et al., 2011, Huxley et al., 2005) and one study found almost half of all patients with PDAC have diabetes at the time of cancer diagnosis. However, individuals over the age of 50 with recent onset diabetes also have an increased chance of a PDAC diagnosis within 2-3 years (Pannala et al., 2009).

PDAC is an aggressive carcinoma that typically presents at an advanced stage and is unresponsive to most treatment modalities (Wolfgang et al., 2013, Ryan et al., 2014). By the year 2030, PDAC is predicted to become the second leading cause of cancer mortality (Rahib et al., 2014). Studies have demonstrated strong genetic alterations associated with pancreatic cancer, with whole-exome sequencing identifying mutations and somatic copy number alterations in key oncogenes and tumour suppressor genes (e.g. *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*) (Jones et al., 2008). Additional whole-exome and

whole-genome studies have also revealed less prevalent alterations in genes such as those coding for regulators of axon guidance (Bailey et al., 2016), and germline mutations in “DNA damage” genes such as *BRCA1*, *BRCA2*, *PALB2* or *ATM* cause genomic instability in a subset of PDACs (Roberts et al., 2016, Sahin et al., 2016).

Some have questioned whether the molecular and genetic changes that occur during early PDAC could also trigger diabetes in a subset of patients (Ben et al., 2011). Diabetes may be a paraneoplastic phenomenon caused by the cancer and secretion of diabetogenic products such as amylin (Permert et al., 1997), and adrenomedullin (Aggarwal et al., 2012b). Interestingly, a resolution of diabetes after PDAC resection is observed in a subgroup of individuals, also supporting the hypothesis that PDAC could be triggering diabetes (Pannala et al., 2008). PDAC and T2D share certain common background risk factors such as hyperinsulinemia, genetic risk, obesity and lifestyle (Perseghin et al., 2012, Kuruma et al., 2014). However, regardless of this, is has been suggested that PDAC could accelerate diabetes by inducing β-cell dysfunction through either the direct destruction of normal pancreatic tissue (advanced stages), the indirect process of pancreatitis caused by impairment of the pancreatic duct system, or from secondary effects related to conditions such as jaundice (Dugnani et al., 2016).

1.4 Type 1 Diabetes

Type 1 diabetes (T1D) affects the lives of an estimated 400,000 people in the UK and is increasing in prevalence at an annual rate of 4% (https://www.diabetes.org.uk/type-1-diabetes, accessed 16/05/2021). T1D exhibits striking heterogeneity across the population with differences in age at onset, disease progression timing and severity. It occurs as a result of both

genetic and environmental factors and individuals with T1D must depend on lifelong administration of exogenous insulin to survive (Leete et al., 2020, Morgan and Richardson, 2018).

T1D is an autoimmune disease that initiates when the immune system selectively targets and destroys the β-cells. The immune assault is dominated largely by CD8+ T cells, but CD4+ T cells and CD20+ B cells are also present and vary in population size depending on the age of the individual at diagnosis (Willcox et al., 2009, Leete et al., 2016). Compared to adults and adolescents, children under 7 years of age have higher numbers of immune cells infiltrating the pancreas and a higher ratio of CD20+:CD4+ cells in the insulitic infiltrates (Leete et al., 2016, Leete et al., 2018). Microscopy studies have demonstrated that the presence of a more aggressive immune assault, as judged by higher numbers of T and B cells infiltrating the islets, results in the presence fewer insulin containing islets (ICIs) at diagnosis. This is likely due to the destruction of the insulin producing cells by the immune cells. Hence, the pancreatic "landscape" of children under 7 years of age is very different to that of children over the age of 13 years, with the under 7's having fewer ICIs. This observation has led to the designation of two specific endotypes of T1D, namely T1DE1 (typically seen <7 years old) and T1DE2 (typically seen >13 years old) (Leete et al., 2020). This observation is important for immunotherapy trials where the effectiveness of targeting CD20 B cells may alter depending on the age at diagnosis, since T1DE2 cases have lower numbers of CD20+ B cells compared to T1DE1 cases. Indeed, there is evidence from clinical trials of less effective responses to anti-B cell therapy (with anti-CD20; Rituximab) in T1D patients diagnosed between ages 8 and 40 (Pescovitz et al., 2014, Dufort et al., 2019). Recently, immune therapy has demonstrated that anti-CD3 antibody treatment

(Teplizumab) can delay the onset of T1D for around 2 years in subjects having islet autoantibodies (Herold et al., 2019).

1.4.1 A brief overview of the genetics of T1D

T1D is associated with over 60 genetic loci, large numbers of which are associated with the Human Leukocyte Antigen (HLA) region on chromosome 6p21 (Bakay et al., 2019). The role of the HLAs is in antigen presentation on immune cells, and immune cell activation. Within Class II HLA genes, particular loci confer higher risk factors for developing T1D. These are the DR and DQ loci, with individuals heterozygous for DRB1*03 and DRB1*04 (DR3/DR4) at highest risk of disease (Noble, 2015). Class I HLA loci also pose a genetic risk for the development of T1D and individuals with an HLA-A*24 allele are found to have reduced residual β-cell function (Nakanishi et al., 1993).

Due to the polygenic nature of T1D, genetic risk scores can be used to assist clinicians in monitoring individuals who are genetically at-risk of developing T1D. The T1D genetic risk score assesses over 60 single nucleotide polymorphisms (SNPs) both within and outside the HLA regions (Oram et al., 2016, Sharp et al., 2019). Genes strongly associated with the development of T1D are mainly expressed in the β-cells (~80%) and top hits include genes involved in interferon (IFN) signalling, JAK1/JAK2/TYK2 function in IFN signalling, and foreign body (e.g. bacteria and viral) pattern recognition receptors (PRRs) (Marroqui et al., 2015).

1.4.2 A brief overview of islet autoantibodies

The appearance of T1D associated autoantibodies in the blood often precedes T1D diagnosis. Autoantibodies are antibodies produced by B cells that recognise the body's (self) endogenous proteins, also referred to as self-recognition. T1D associated autoantibodies include β-cell derived insulin (IAA),

glutamic acid decarboxylase 2 (GAD65), protein tyrosine phosphatase like protein (IA-2), zinc transporter 8 (ZnT8) and tetraspanin protein family member 7 (TSPAN7) (Palmer et al., 1983, Rabin et al., 1992, Baekkeskov et al., 1990, Wenzlau et al., 2007, McLaughlin et al., 2016). There are strong genetic links to development of islet autoantibodies and diabetes associated HLA loci. In particular, GADA and IAA are associated with the DR3 and DR4 haplotypes (Giannopoulou et al., 2015). A study published in 2013 proposed that over 90% of the children sampled had tested positive for autoantibodies before the diagnosis of T1D (Wenzlau and Hutton, 2013). Additionally, an individual is found to be at greater risk of developing T1D if they seroconvert to multiple autoantibodies (Lampasona and Liberati, 2016). The development processes underlying seroconversion to islet autoantibodies, and the role these autoantibodies play in disease progression has yet to be fully elucidated, however, their significance in diagnosing T1D is very clear.

1.4.3 Viral triggers of T1D

The annual increase in children diagnosed with T1D seen in many populations cannot be explained by genetics alone, and environmental factors have long been implicated as contributors to disease development. Viruses are believed to trigger T1D, and decades of research has drawn links between T1D and the *Picornaviridae* family (Richardson et al., 2009, Krogvold et al., 2015a, Richardson and Morgan, 2018, Rodriguez-Calvo, 2019). Enteroviruses are a common subtype of *Picornaviridae* and are spread via human-to-human transmission. They are a non-enveloped, single-stranded positive-sense RNA group of viruses. Found globally, these viruses can transmit rapidly in young children, often without symptoms but can lead to serious conditions such as myocarditis (Rodriguez-Calvo, 2019).

The development of T1D has been associated with enteroviruses since 1969, when higher antibody titres of Coxsackie B virus (CBV), a subtype of enteroviruses, were found in individuals recently diagnosed (<3 months) with T1D (Gamble et al., 1969) compared to controls or subjects diagnosed > 3 months. The development of islet autoantibodies is also associated with CBV infection, with CBV B1 and B5 found in association with IAA (Sioofy-Khojine et al., 2018). Viral infections have not been found in association with GADA, but IAA and GADA are found on different HLA haplotypes so it may be the case that particular haplotypes of HLA (e.g. HLA-DR4) may enhance susceptibility to viral triggered T1D. Conversely, it cannot be ruled out that cellular changes triggered by viral infection could influence the development of IAA antibodies. The cellular receptor hijacked by Coxsackie viruses for cellular entry, namely the Coxsackie and Adenovirus receptor (CAR), colocalises with insulin secretory granules in β-cells (Ifie et al., 2018). It could be speculated that this receptor localisation could contribute to the association seen between IAA and Coxsackie infection, since IAA is associated with insulin granules while GADA is not. However, cases have demonstrated that CBV1 has not always been found in association with a particular HLA genotype (Oikarinen et al., 2014).

The link between T1D and enterovirus infections has also been observed in epidemiological studies. For example, the Type 1 Diabetes Prediction and Prevention (DIPP) study found evidence of enteroviral shedding in stool samples collected from children before the detection of autoantibodies. However, they also found that some Coxsackie viral types (CBV3 and CBV6) were non-pathogenic and associated with a reduced risk of islet autoimmunity (Honkanen et al., 2017). The Diabetes and Autoimmunity Study in the Young (DAISY) was a major study established to monitor genetically predisposed

children for autoantibody detection and T1D diagnosis. Once autoantibodies were detected, blood or rectal swabs were collected every 3-6 months, which revealed the presence of enteroviral RNA in those diagnosed with early T1D (Stene et al., 2010). This suggests that an enteroviral infection may contribute to the development of T1D by either triggering or augmenting autoimmunity in some children.

Another recent epidemiological study, the Environmental Determinants of Diabetes in the Young (TEDDY) looked at persistent Coxsackie B virus infection in young children and found an association between development of islet autoimmunity and persistent viral infection (Vehik et al., 2019). Further evidence for the role of persistent viral infection in T1D comes from a large meta-analysis of molecular studies which found a significant association between enterovirus and T1D. The probability of detecting enterovirus infection in recent onset diagnosis in children was ten times higher than non-diabetic children. Enteroviruses were also detected in children with established diabetes, indicating a potential persistence within the pancreas (Yeung et al., 2011, Kim et al., 2019).

Histology samples have also demonstrated that Coxsackie virus VP1 protein can be detected in the islets of people with T1D up to a duration of ten years after diagnosis, indicating viral persistence (Dunne et al., 2019). The CBV VP1 protein is a capsid protein that’s coded for several functions, including inducing cell cycle arrest (Wang et al., 2019). Molecular studies have demonstrated that persistence of the virus in the pancreas may be promoted by a 5’ deletion of the viral genome, which attenuates replication and lytic capability (Bouin et al., 2019, Bouin et al., 2016).

Much of our knowledge of viral triggered diabetes has come from epidemiological studies because research conducted using human pancreatic samples is limited. This is due to restricted access to pancreatic tissue and human islets, especially from individuals with recent onset diabetes. However, a few groups have been able to successfully analyse evidence of viral infection in the pancreatic landscape. The Diabetes Virus Detection (DiViD) study sourced pancreas tissue from six recently diagnosed living individuals (<9 weeks after diagnosis) and found that viral protein VP1 was present in islet cells in all six cases. Additionally, enteroviral RNA was found to be present in islets from four of the cases (Krogvold et al., 2015a). Richardson et al. (2009) also detected VP1 in the islets of 61% (44/72) of young cadaver donors that had recent onset T1D compared to 6% (3/50) of neonatal controls (Richardson et al., 2009).

Despite the substantial amount of evidence suggesting an association between T1D and enteroviral infection, it has yet to be shown that viruses can directly trigger T1D in primate models or in humans. One theory is that the genetic predisposition to T1D may also act as a platform for increased susceptibility to viral infection. A histology study found that insulin containing islets (ICIs) associated with viral VP1 protein were generally (>60%) non-inflamed, meaning there were low levels of insulitis (Willcox et al., 2009) potentially indicating that viral infection precedes insulitis and β-cell destruction. There is also the question of why some genetically suspectable children become infected with Coxsackie virus but do not go on to develop T1D. Despite these outstanding questions, the manufacturing of vaccines against enteroviruses have progressed forward and ProventionBio™ has developed a vaccine against Coxsackie virus with the hope that it will protect at-risk children from developing

T1D: at-risk being defined as individuals with T1D-associated HLA loci and those with a first-degree relative with T1D.

More recently, a possible link has been made between COVID-19 and the development of T1D in children. Over thirty children in hospitals across London, UK, presented with new-onset T1D during the peak of the COVID-19 pandemic, approximately double the number of cases seen in previous years (Unsworth et al., 2020). However, cross-sectional data from Italy showed a decrease in new-onset cases, but this may have been a result of delayed access to healthcare settings (Rabbone et al., 2020). Studies are ongoing to investigate possible links between COVID-19 and development of T1D.

1.4.4 Other triggers of T1D

1.4.4.1 Cow's milk

For decades, researchers have drawn links between the development of T1D and the introduction of cow's milk into the diet (Borch-Johnsen et al., 1984, Virtanen et al., 1991). The DAISY study (as mentioned above) explored this theory further and demonstrated no association between cow's milk, the HLA-DR3/4 genotype and progression to T1D. However, a low/moderate risk association was observed between cow's milk and other T1D associated HLA genotypes (Lamb et al., 2015). These results closely followed a study that found early exposure to cow's milk could alter the adaptive immune responses of children who developed T1D. One hypothesis put forward to explain the potential link is that particular proteins in cow's milk, such as bovine insulin, may mimic human proteins at the molecular level, leading to an immune reaction against the milk (Vaarala, 2002, Paronen et al., 2000). However, the Type 1 Diabetes Prediction and Prevention Project (DIPP) found no association

between β-cell autoimmunity and introduction of cow's milk into a child's diet (Virtanen et al., 2006).

1.4.4.2 Vitamin D

A deficiency in vitamin D has been suggested to increase the likelihood of developing T1D in at-risk children. This observation stems from linking low sunlight hours to incidence rates of T1D in Finland, which has the highest number of T1D cases globally (Dahlquist and Mustonen, 1994). However, others have highlighted that populations across the border in Karelian Republic of Russia have similar HLA risk genotypes and similarly few sunlight hours but lower rates of T1D (Kondrashova et al., 2005). This poses the question as to the true significance of vitamin D in the development of T1D, but data suggest that vitamin D supplementation in early childhood may have protective affects against T1D (Stene et al., 2000, Hyppönen et al., 2001, Harris, 2002, Zipitis and Akobeng, 2008).

A range of observations have been reported in clinical studies analysing the effects of vitamin D supplementation in patients with T1D. Some report increases in regulatory T cells in conjunction with a decrease in HbA1c levels (Gabbay et al., 2012, Treiber et al., 2015, Bogdanou et al., 2017, Panjiyar et al., 2018). However, opposite effects have been shown by others (Pitocco et al., 2006, Bizzarri et al., 2010, Haller et al., 2013, Perchard et al., 2017, Sharma et al., 2017). Sources of discrepancy in the data may come down to differences in molecular forms and concentrations of vitamin D used in the various studies, and investigations into the impact on vitamin D are still ongoing.

1.4.4.3 Gut microbiota

Some have proposed that reduced exposure to infections through improved hygiene and medical practise has led to an increase in allergies and

inflammatory diseases such as T1D (Jakobsen and Szereday, 2018, Bach, 2018). Interestingly, the diversity and composition of microbiota found within the gut has been found to differ between individuals with T1D and control subjects. The microbiome plays an influential role in shaping the immune system, which is why it has come into focus for diabetes research (Koenig et al., 2011). Children with T1D display microbiomes containing higher levels of Bacteroidetes phyla and lower levels of Firmicutes phyla, but the significance of this has yet to be fully elucidated (Boerner and Sarvetnick, 2011). A second study found no differences in bacterial populations between diabetic and non-diabetic children, but these children were controlled for stable glycaemic control and physical activity, which may have impacted the findings (Stewart et al., 2017).

While these studies may highlight interesting differences seen in the microbiomes of individuals with diabetes, the clinical translation of results are not clear and altered bacterial profiles may be a secondary effect of the development of T1D rather than a linked cause. Further longitudinal studies are needed for investigation into the role of gut microbiota in T1D.

1.5 The pancreas in health and disease

1.5.1 Structure and function

The pancreas is the eighth largest organ in the body and located behind the stomach in the upper abdomen. During the embryonic stages of pancreatic development, the early pancreas forms from buds stemming off the gut tube at a point ventral to the appearance of the liver. A dorsal bud is followed by two ventral buds, which fuse together at around 6 weeks gestation age (Pan and Brissova, 2014). The pancreatic head, neck, body and tail form from the dorsal bud while the inferior section of the pancreatic head forms from the ventral bud

(Van Hoe and Claikens, 1999, Pan and Brissova, 2014). Running along the length of the pancreas is the pancreatic duct, which originates in the tail of the pancreas and connects to the common bile duct, draining into the duodenum (Figure 1-1) (Henry et al., 2019). The pancreas has both exocrine and endocrine tissue, meaning excretion of molecules occurs both within the ducts and into the blood respectively (Figure 1-1). The main cell types found within the exocrine region are acinar cells and pancreatic ductal cells. Acinar cells synthesize and secrete digestive enzymes and are located around central lumens while ductal cells form the structure of the duct walls. Digestive enzymes are secreted from the exocrine tissue towards the pancreatic ducts together with water and bicarbonate ions. Enzymes which reach the duodenum include elastase, amylase, lipase, carboxypeptidases, trypsinogen, phospholipases A and chymotyrpsinogen.

The endocrine regions of the pancreas are composed of structures called islets of Langerhans, named for Dr. Paul Langerhans who first described them in 1868 (Langerhans, 1868). These cell structures were found to be responsible for the control of blood glucose within the body and are composed of at least five different endocrine cell types α-, β-, δ-, PP- and ε-cells (Figure 1-1) (Brissova et al., 2005, Ekblad and Sundler, 2002, Arrojo e Drigo et al., 2015, Roscioni et al., 2016). Each of these cell types is distinct in character and role within the endocrine tissue, secreting specific hormones to regulate levels glucose, metabolic hormones and appetite. β-cells principally secrete insulin while α-cells secrete glucagon, and these two hormones work in opposition to regulate blood glucose levels within the body. The δ- cells secrete somatostatin, a hormone that regulates the secretory activity of α-and β-cells, while the γ-cells secrete a polypeptide which is believed to regulate food intake and gastric

emptying (Katsuura et al., 2002). The ε-cells secrete ghrelin and amylin, but the exact functions of these hormones are still uncertain (Kruger et al., 1999).

Compared to the exocrine tissue, islets are highly vascularised and receive 10 times more blood to allow for nutrient and hormone exchange (Jansson and Hellerström, 1983). Up to five arterioles supply blood to each islet and these further branch into capillaries (Ballian and Brunicardi, 2007), leading eventually to the hepatic portal vein (Rorsman and Ashcroft, 2018). The central nervous system regulates islet blood flow, particularly the vagus nerve which, upon stimulation, leads to islet vasodilation and increases in blood supply to the islet when glucose levels are elevated (Ballian and Brunicardi, 2007). This allows for the heightened capacity for endocrine cells to sense glucose and secrete insulin.

The function of each cell type and respective hormone is described in Table 1-1. β-cells are the most studied cell type found within the islets of Langerhans and comprise 28-75% of each islet, while α-cells comprise 10-65% (Brissova et al., 2005). Interestingly, the shape and composition of islets of Langerhans differ across mammals, especially when comparing primate and rodent models (Cabrera et al., 2006). In mouse models, the β-cells are clustered at the core and surrounded by a ring of α-and δ-cells , while in primate models the different cell types are more integrated together in clusters (Cabrera et al., 2006).

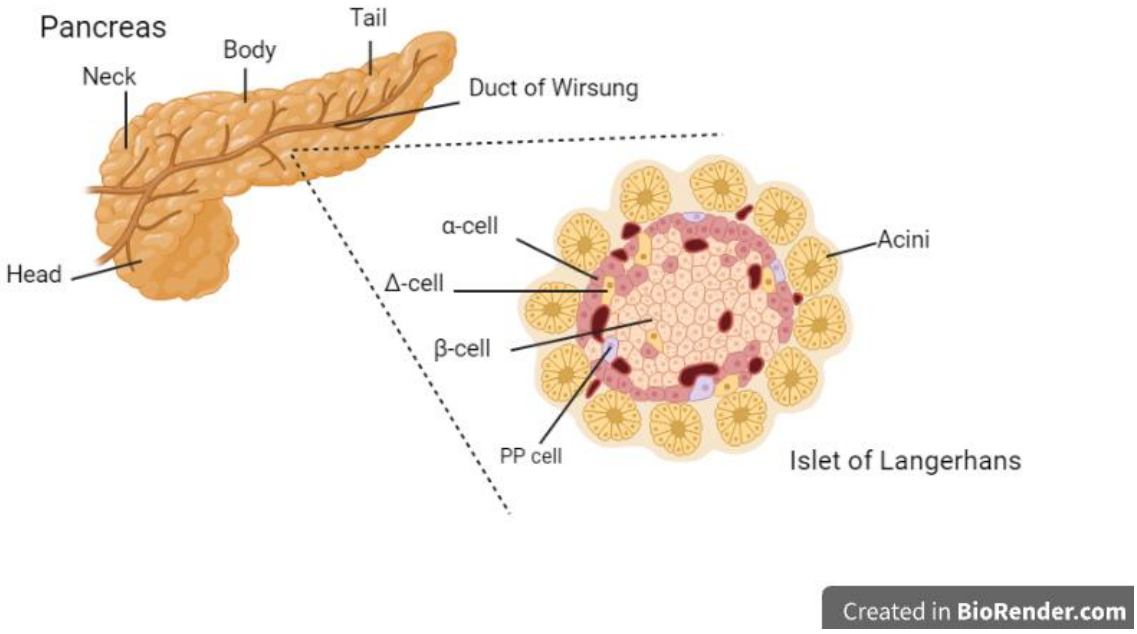


Figure 1-1 Key anatomical features of the human pancreas. Schematic representation of the pancreas demonstrating the exocrine and endocrine regions. Diagram of an islet of Langerhans showing four endocrine cell types. Diagram created using Biorender.

Cell Type	Hormone	Function	Target Organ
α-cell	Glucagon	Raises blood glucose levels	Liver and adipose tissue
β-cell	Insulin	Lowers blood glucose levels	Liver then skeletal muscle and adipose
δ-cell	Somatostatin	Inhibits secretion of insulin and glucagon	α-cell and β-cell
PP-cell	Pancreatic Polypeptide	Suppression of secretion by exocrine pancreas and bile secretion	Pancreas
ε-cell	Ghrelin	Stimulates appetite and suppresses insulin release	Brain, pancreas

Table 1-1 Endocrine cell types present in the pancreas and their function

Islet topography is important for studying cell-to-cell interactions and events that occur through gap junctions and paracrine signalling. These mechanisms differ across species and variability in islet topography may be the causative factor. For example, in rodents oscillations in membrane potential are coordinated in β-cells, while they are less well synchronised in humans. This could also help to explain why we see differences in intracellular calcium levels and insulin secretory profiles across species (Cabrera et al., 2006). The full explanation as to why rodent and adult human islets differ in appearance has yet to be discovered but may reflect evolutionary adaptation or environmental pressures.

The islets of Langerhans are surrounded by extracellular matrix, consisting of basement membrane and interstitial matrix (Korpos et al., 2013). This layer protects the islets from immune cell invasion and supports islet architectural integrity. The basement membrane is mainly composed of glycoproteins while the interstitial matrix consists of fibrillar collagens. Across the extracellular matrix, compounds such as laminins, collagens, heparan sulphate and proteoglycans can be found (Bogdani et al., 2014, Korpos et al., 2013).

1.5.2 Insulin synthesis and processing

The gene for insulin, *INS*, is located on chromosome 11. The transcription and translation of the insulin gene is regulated by blood glucose levels (Poitout et al., 2006). The *INS* gene is controlled mainly by transcription factors MafA, PDX1 and NeuorD1 (Andrali et al., 2008, Tokarz et al., 2018) and PDX1 binding to the *INS* gene promoter is partly controlled by elevated glucose concentration (Poitout et al., 2006). After transcription, translation occurs and results in a precursor molecule called preproinsulin (Patzelt et al., 1978, Tokarz et al., 2018, Guest, 2019). Translation to preproinsulin begins in the cytoplasm but when passing through the rough ER, cleavage occurs on the N-terminal signal

peptide via a signal peptidase to form proinsulin (Patzelt et al., 1978). Once in a proinsulin form molecular folding occurs, and the A and B chains of proinsulin are linked by disulphide bonds. This triggers further transport through the Golgi for sorting into immature secretory granules rich in Zn^{2+} and Ca^{2+} (Tokarz et al., 2018, Guest, 2019). The immature secretory granules become more acidic during the processing of proinsulin and eventually C-peptide is cleaved by the prohormone convertases PC1/3, PC2, and carboxypeptidase E (CPE) (Guest, 2019). Basic amino acids located at the C-terminus of cleaved polypeptides resulting in the mature form of insulin, containing A and B chains linked by disulphide bridges (Tokarz et al., 2018, Hutton, 1994). In its intracellular secretory form, insulin is not very soluble and detected in crystallised form, but upon exocytosis into the extracellular space, insulin becomes more soluble and dissolves to produce insulin monomers, which can act on target tissues. Previous studies have estimated that one β-cell may contain >10,000 insulin granules (Rorsman and Renström, 2003).

1.5.3 Glucose induced insulin secretion

β-cells do not have membrane-bound glucose receptors, so glucose enters the β-cell through facilitated diffusion via insulin-independent glucose transporters (GLUT-2 in rodents; GLUT-1, 2 and 3 in humans) (Figure 1-2) (De Vos et al., 1995). After entering the cell, glucose triggers metabolic pathways to induce insulin secretion. In brief, glucose is metabolised through the process of glycolysis, beginning with glucokinase (or hexokinase in other cell types) catalysing the phosphorylation of glucose to glucose – 6 – phosphate (Middleton, 1990). Compared to other cell types, higher concentrations of glucose are needed to trigger a response from glucokinase in β-cells because insulin is restricted for release during conditions when glucose levels are

elevated beyond basal levels. This acts as a control mechanism for the release of insulin. At the end of the glycolysis cycle, pyruvate is formed and enters the tricarboxylic acid (TCA) cycle in the mitochondria, leading to the generation of NADH. NADH is a reducing agent that drives the electron transport chain to create ATP, resulting in a higher cytosolic ATP/ADP ratio. This causes the closure of ATP-sensitive K+ (K_{ATP}) channels by binding to Kir6.2 subunits. Upon closure, the cell enters a state of depolarisation and activation of L-type voltage-dependant calcium channels (VDCCs), which allows calcium (Ca^{2+}) to enter the cell. The influx of calcium activates exocytosis of secretion-ready insulin granules (Fu et al., 2013, Sabatini et al., 2019).

Exocytosis in β-cells occurs in a similar fashion to the soluble N-ethlymaleimide-sensitive factor attachment protein receptor (SNARE)-regulated synaptic vesicle release. Fusion occurs between the insulin containing vesicles and plasma membrane and requires the assembly of a SNARE protein complex (syntaxin, synaptosomal-associated protein of 25kDa (SNAP-25)), and vesicle-associated membrane protein 2 (VAMP-2) (Rorsman and Renström, 2003). The insulin containing vesicles dock at the plasma membrane and fusion of the membranes occurs after localised elevation in calcium concentration. Synaptotagmin proteins V or VII of the SNARE complex are responsible for sensing Ca2+ (Sabatini et al., 2019). The GTP-binding protein Rab3A interacts with the vesicle bound Rab3-interacting molecule (Rim) and helps the SNARE complex to interact with synaptotagmin, as well as the Ca^{2+} channels (Rorsman and Renström, 2003).

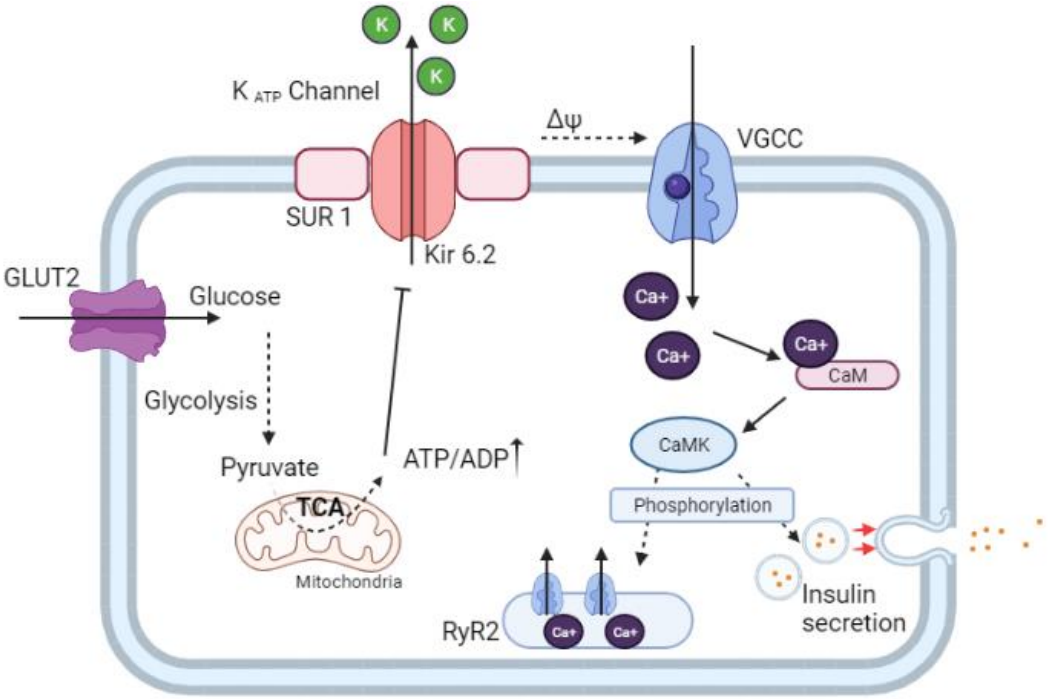


Figure 1-2 The K_{ATP} channel-dependent pathway of glucose-induced insulin secretion. Glucose enters the cell via GLUT2 transporters and is phosphorylated by glucokinase. Phosphorylated glucose is then processed through glycolysis to pyruvate, which is converted to ATP in the mitochondria. Increase in the ATP/ADP ratio leads to the closure of K_{ATP} channels via binding to their Kir6.2 subunits, and resultant depolarisation (Ψ) of the cell. Opening of the voltage-gated Ca^{2+} channels (VGCC) and influx of calcium causes a rapid increase in [Ca^{2+}], which interacts with Ca^{2+} sensing proteins within SNARE complexes (synaptotagmins) to facilitate fusion between plasma membrane and insulin containing vesicles. Diagram created with Biorender.

Insulin secretion can be upregulated through increased levels of cyclic adenosine monophosphate (cAMP), in a glucose-dependent manner. A rise in cAMP occurs through activation of Gs-protein-coupled receptors (GPCRs) in response to incretin hormones such as glucagon-like-peptide 1 (GLP1) and gastric inhibitory polypeptide (GIP) (Figure 1-4). In response to glucose, GLP1 and GIP circulate from the gut and help regulate insulin release after food intake (MacDonald et al., 2002). Muscarinic Gq-protein coupled receptors are also involved in promoting insulin secretion and molecules like acetylcholine bind to these receptors, which leads to second messenger activation and downstream effects to activate protein kinase C (PKC) and promote insulin granule release. Certain second messengers can also bind to the ER membrane, triggering a release of calcium from the ER stores and promoting insulin granule release (Turk et al., 1993, Yamazaki et al., 2010, Ahrén, 2000).

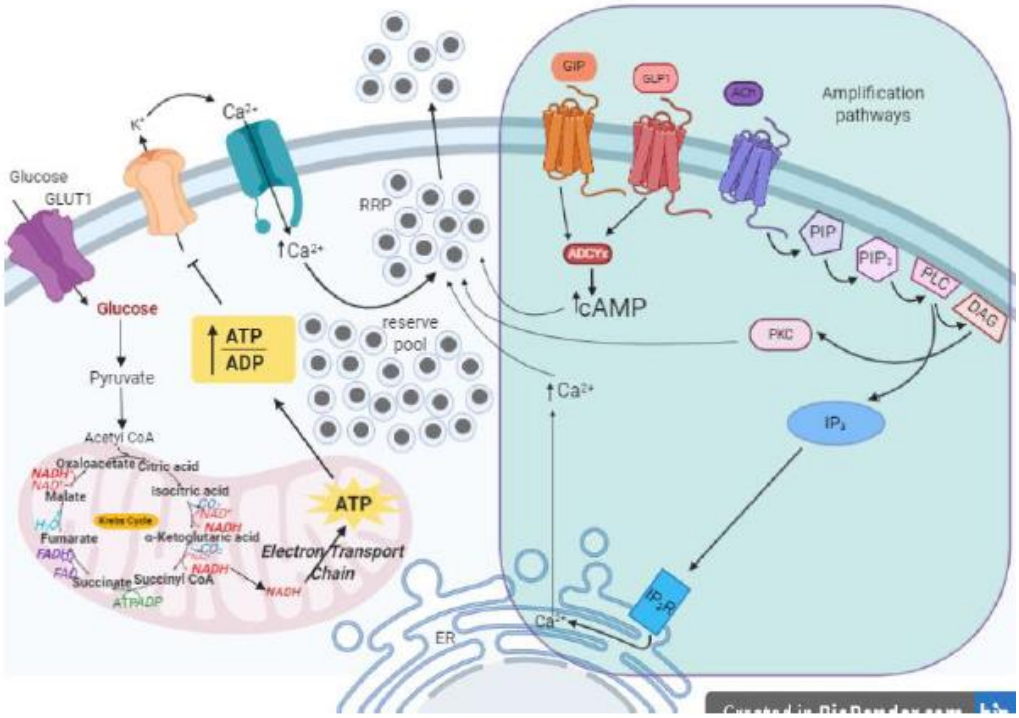


Figure 1-3 Pathway of insulin secretion.

Glucose approaches the cell and enters via the glucose transporter GLUT1. Glucose is metabolised though the glycolysis cycle, which creates pyruvate. Pyruvate enters the Krebs cycle, which leads to the production of NADH, a reducing equivalent that drives the electron transport chain to produce ATP. Increases in the ATP:ADP ratio closes the ATP sensitive potassium channels (K_{ATP}) on the cell membrane, resulting in depolarisation of the cell membrane. This leads to the opening of voltage gated calcium channels and subsequent influx of calcium. Changes in calcium levels trigger exocytosis of insulin containing granules. Insulin secretion can also be altered by increases in intracellular cAMP via Gs-protein coupled receptor (GPCR) signalling (e.g. incretin hormones GIP and GLP-1 binding to cell membrane receptors). Insulin secretion can also be influenced by Gq-coupled protein receptors (e.g. acetylcholine) which results in an increase in phospholipase C (PLC) activity, which increases second messengers diacylglycerol (DAG) and inositol

triphosphate (IPS). DAG increases PKC activity and IP3 binds IP3 receptors on the ER membrane which releases Ca^{2+} from the intracellular ER stores into the cytoplasm. These mechanisms augment insulin secretion, however, are insufficient to trigger insulin secretion in the absence of glucose. Image courtesy of Dr. Jessica Chaffey (with permission) and created on Biorender.

1.5.4 Dynamics of insulin release

Glucose-stimulated insulin secretion occurs in a characteristic biphasic pattern and consists of rapid first phase followed by a more gradual, sustained second phase (Grodsky, 1972). Secretion of insulin vesicles in the first phase occurs from 'readily releasable pools' (RRP) (Figure 1-3). These granules (5-15% of all granules) contain mature insulin and are docked close the plasma membrane (Hou et al., 2009, Kalwat and Cobb, 2017). Once the RRP granules are depleted, the rate of granule release decreases as granules are recruited from intracellular reserve pools. This second pool of granules undergo ATP-, Ca^{2+}, time/temperature-dependent 'priming' reactions (Rorsman and Renström, 2003) for the second phase of insulin secretion. Both phases follow a similar pattern of events within the β-cell; glucose entry, increased metabolic activity, closure of K_{ATP} channels, calcium influx and granule transport. In addition to the K_{ATP}-dependent pathway, other cellular signalling pathways and second messenger pathways have been implicated in the phased action of insulin secretion (Hou et al., 2009).

Insulin output from β-cells can also be regulated by hormones such as somatostatin and adrenaline, which are delivered to cells through circulation or released by adjacent cells within the islets. This can cause a paracrine modulation of insulin secretion. Insulin secretion may also be modified by other neurotransmitters and gastrointestinal incretins (Jones and Persaud, 2010).

1.5.5 Insulin action and storage

Once insulin is released from the β-cells, it acts on insulin-sensitive tissues, specifically through heterotetrameric insulin receptors of the tyrosine kinase family. Upon insulin binding to a receptor, a conformational change occurs within the extracellular α-subunits of the receptor, triggering the activation of intracellular β-subunits via phosphorylation of tyrosine residues (Schlessinger, 2000). The insulin receptor substrates (IRS) are made up of four main members (Burks and White, 2001) IRS 1 to 4. The first two (IRS-1, IRS-2) are ubiquitously expressed, IRS-3 is mainly expressed in adipocytes, and IRS-4 can mainly be found in the CNS (Fantin et al., 1999, Lavan et al., 1997, Sun et al., 1995). Tyrosine phosphorylation leads to the recruitment of downstream effectors by IRS proteins. IRS-1 and IRS-2 both dock with the regulatory subunit p85 of class IA phosphatidylinositol 3-kinase (PI3K), followed by the recruitment and activation of catalytic subunit p110α of PI3K. This complex catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) in the plasma membrane (Cantley, 2002). PIP3 activates phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate the serine-threonine kinase Akt/PKB. PDK1 and a protein called transducer of regulated CREB activity 2 (TORC2) stimulates Akt/PKB to phosphorylate a broad range of proteins (ASK160, GSK3, mTORC1) that ultimately lead to cellular responses to insulin (Manning and Cantley, 2007).

1.5.6 Regulation of glucose storage by insulin

Healthy individuals have a varied feeding and fasting pattern throughout the day and to accommodate this pattern the body closely regulates plasma glucose levels within a range of 3 to 8 mM. This process is managed by the central nervous system (CNS), liver, gastrointestinal tract, skeletal muscle, adipose tissue and endocrine pancreas. Insulin secretion is stimulated by release from

the β-cells by increasing levels of plasma glucose, neuronal signals from the parasympathetic nervous system and non-nutrient secretagogues. This process is vital because insulin acts as the primary regulator of blood glucose and directs glucose uptake by muscle and adipose tissue. It also promotes glucose storage in the liver, muscle and adipose tissue, and influences the inhibition of glucose production from the liver.

1.6 How can we study the pancreas in the context of T1D?

1.6.1 Animal models

Rodent models of T1D have been used to understand disease processes for decades. The most common rodent models of T1D include the non-obese diabetic (NOD) mouse and the bio-breeding diabetes-prone (BB) rat. Important insights into T1D and the pathology of the pancreas have been gained by longitudinal studies and early pancreatic development. However, despite the great advancements made, rodent models do not accurately reflect the situation in human pancreas, the most prominent example being the different arrangement and topography of the islets. The inflammatory profile and progression of insulitis is also quite different in rodent models vs humans, as infiltration of the immune cells into rodent islets appears to be more pronounced (In't Veld, 2014). Therefore, there are limitations to what we can hope to understand about the initial stages of disease during the points in which an immune assault on β-cells is initiated. There are several ways to 'cure' or reverse diabetes in rodent models, however these have been unsuccessful to date in reversing diabetes in humans, including the various immunotherapies (Ben Nasr et al., 2015, Lenzen, 2017). For this reason, it is imperative that studies are performed using human material and/ or relevant human model cell lines.

1.6.2 Tissue biobanks and human pancreatic samples

Access to human pancreatic material for the purposes of research can be quite restricted. While there are legislative reasons for this in the UK, other factors affecting tissue availability include positioning of the pancreas within the body and the risks of biopsy to normal physiological functions. The pancreas is found within the middle of the abdominal cavity and behind the stomach, which can be difficult to access through surgical routes. Serious illness can result if pancreatic digestive enzymes leak into the cavities following improper sealing during biopsy.

As such, the availability of pancreatic tissue is limited and even more so from individuals with T1D (<600 across all cohorts) (Morgan and Richardson, 2018). For my thesis, I accessed three collections which I briefly summarise below.

1.6.3 EADB

University of Exeter, UK hosts the Exeter Archival Diabetes Biobank (EADB). This collection was initially established by Professor Alan Foulis who collected post-mortem human pancreatic material for the purposes of research (Foulis et al., 1986, Morgan and Richardson, 2018). Limitations of this collection include its historical age (collected >30 y ago), variable quality of post-mortem tissue and non-standard fixation techniques. However, this collection is unique in that in holds the largest number of pancreatic tissue samples from children under the age of ten with recent onset T1D (Morgan and Richardson, 2018). Fortunately, improved medical treatment means that T1D is rarely a cause of death for young children today, so the EADB collection is a rare repository for studying the immune pathology of early T1D. This collection also houses a large number of samples from individuals without diabetes collected over the same time period.

1.6.4 nPOD

The Juvenile Diabetes Research Foundation (JDRF) established the Network for Pancreatic Organ Donors with Diabetes (nPOD) biobank in 2007 to address the need for pancreatic tissue samples. This collection houses material from donors with T1D, non-diabetic autoantibody positive individuals, autoantibody negative non-diabetic individuals and people with other β-cell disorders. The nPOD biobank also contains samples of spleen, intestine, serum, blood and lymph-nodes (Campbell-Thompson et al., 2012). Analysis has been conducted on each of the donor samples to gather insights on autoantibody status, C-peptide levels and HLA genotype. Of the 514 donors available (aged 4.4 – 93 years), 167 are classified as having T1D.

1.6.5 DiViD

The Diabetes Virus Detection Study (DiViD) collection hosts samples of pancreas tail from biopsies of living participants (3 adult male and 3 adult female aged 24-35) who were newly diagnosed with T1D (3-9 weeks of diagnosis). This study exemplifies the challenges with collecting pancreatic samples as further collections were cancelled due to post-operative complications (Krogvold et al., 2014). For study controls, samples analysed from this collection are often compared to non-diabetic controls in the nPOD collection because they have comparable tissue processing standards and patient ages.

1.6.6 *in-vitro* β-cell models

The gold standard for the study of islets *in vitro* is to use isolated human islets from organ donors, but these studies can be fraught with limitations. Human islets used in research in the UK are made available only after they are designated as unsuitable for clinical transplant into patients. As they come from a wide range of organ donors, the health status of the individual (e.g. sex, BMI,

health conditions, age) and condition of the islets at end of life may affect experimental results. Also, primary islet cells do not replicate in the laboratory (Nano et al., 2019).

1.6.7 Clonal β-cell lines

Due to the challenges outlined above, research on diabetes relies on clonal cell lines to pursue scientific questions. However, numbers of pancreatic clonal lines are few, particularly for human pancreatic β-cells lines. Over the last decade, the two most commonly used β-cell lines are EndoC-βH1 (Ravassard et al., 2011) and 1.1B4 cells (McCluskey et al., 2011). At the time of their original description and release, the 1.1B4 cells were considered to resemble human β-cells, albeit with low insulin content. However, as I explain in later chapters, our group discovered this to be incorrect.

The EndoC-βH1 cells are considered to physiologically mimic β-cells but are quite difficult to use in the laboratory and genetic manipulation by transfection can be problematic. Additionally, researchers must undergo training to successfully propagate these cell lines (Ravassard et al., 2011). Advancements have been made on the EndoC-βH1 cell line, with recent versions EndoC-βH2, EndoC-βH3 and ECN90 becoming available (Scharfmann et al., 2014, Benazra et al., 2015). These improvements resulted in cell lines with decreased cell proliferation but increased β-cell gene expression. For example, EndoC-βH3 is tamoxifen-sensitive and responds to this agent by arresting proliferation and increasing insulin expression (Benazra et al., 2015).

There are several rodent clonal cell lines also available for research in diabetes, and these include RINm5F, MIN6, INS-1, INS-1 832/13 and BRIN-BD11. Some of these cell lines are reliably glucose responsive and thus useful for insulin secretion studies. They have also been extensively used to research β-cell

ontogeny. Interestingly, most clonal β-cell lines can form pseudoislets, which are 3D aggregates of cells. The formation of these pseudoislets can improve the physiological similarities to β-cells in terms of gene expression and insulin secretion (Persaud et al., 2010).

1.6.8 Stem cell derived β-cells

Stem cells are an important area of research, not just for providing cellular models for diabetes research but also for engineering innovative ways of replacing patient β-cells with stem cell-derived β-cells. A study in 2014 demonstrated that pluripotent stem cells could differentiate into β-cell like cells (Rezania et al., 2014). The potential to regenerate β-cells from stem cells is promising, but hurdles need to be overcome in developing models that will differentiate into mature β-cells, instead of halting at the immature cell stage, as is often seen in these studies (Odorico et al., 2018, Balboa et al., 2019). Pluripotent stem cells have also provided a fruitful source of information on neonatal diabetes. They have provided a model for identifying mutations in the *INS* gene that alter cellular function leading to disease (Balboa et al., 2018). As CRISPR-cas9 technology continues to expand, there are further opportunities to gain insight from pluripotent stem cells through faster genetic manipulation of target genes (Balboa et al., 2018).

1.7 The PD-1 / PD-L1 inhibitory pathway and its role in diabetes

The immune system has evolved to eliminate almost any threat from foreign organisms. Through the combined effector potentials of CD4+ and CD8+ T cells, B cells, and innate immune cells, the immune system can coordinate and deliver potent damage to invaders. However, the immune system must do so while sparing healthy cells and maintaining self-tolerance. Self-tolerance broadly refers to a breadth of mechanisms used by the body to limit and prevent

the destruction of self-tissues by self-reactive T and B cells. This process is carried out by numerous checks and balances on immune responses that function during lymphocyte development. The PD-1/PD-L1 pathway is one such checkpoint and negatively regulates CD8+ T cell activation and function. However, perturbing these self-tolerance processes can profoundly impact host physiology and lead to life-threatening autoimmune diseases such as T1D, pneumonitis thyroiditis, dermatitis thyroiditis, and hypophysitis (Sharpe and Pauken, 2018).

For T cells, initial antigen-mediated activation involves peptide-MHC engagement of the T cell receptor (TCR) and positive costimulatory signals such as CD28 on T cells and CD80 on APCs. During this time, negative regulators of T cell activation are also induced to counteract the activation process. Cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1) are some of the first of these regulators to be induced and become active upon engagement with their ligand binding partners (Sharpe and Pauken, 2018).

1.7.1 PD-1

PD-1 is a ‘coinhibitory’ receptor that functions as an immune checkpoint for effector T cells to pass in order to exert full T cell function. Without PD-1, excessive immune-mediated tissue damage can occur (Wykes and Lewin, 2018). PD-1 was discovered in 1992 after the cloning of the PD-1 gene from immune cell lines undergoing apoptosis. PD-1 is also known as CD279 and is composed of a 288 amino acid type 1 transmembrane protein receptor found on all T cells during initial antigen-mediated activation through the TCR (Agata et al., 1996). Furthermore, PD-1 is expressed by subsets of tolerant T cells, regulatory T cells, T cell follicular helper cells, T follicular regulatory cells and

memory T cells, as well as B cells, natural killer (NK) cells, myeloid cells and some cancer cells. (Keir et al., 2008, Ishida et al., 1992, Sharpe and Pauken, 2018).

Located on chromosome 2, the PDCD1 gene that encodes PD-1 is 55-kDa in size with 5 exons (Ishida et al., 1992, Keir et al., 2008). PD-1 is structurally similar to the CD28 family of protein receptors, with an immunoglobulin V (IgV)-like extracellular domain, a transmembrane domain, and an intracellular domain with 2 phosphorylation sites situated in an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Figure 1-4). Following engagement with a ligand, phosphorylation occurs at the two tyrosine residues which leads to the binding of protein tyrosine phosphatases (PTPs), such as Src homology phosphatase-1 (SHP-1) and SHP-2 (Paterson et al., 2011, Keir et al., 2008, Ishida et al., 1992). PTPs can antagonise positive signals that occur through the TCR and CD28, affecting downstream signalling leading to decreased T cell activation and proliferation (Riley, 2009).

On activated B and NK cells, PD-1 activation leads to the inhibitory action of their lytic functions (Terme et al., 2011, Fanoni et al., 2011), while on regulatory T cells (Treg), PD-1 expression can lead to inhibition of immune activity by expression of forkhead transcription factor FOXP3 or release of inhibitory cytokines (e.g. TGFB, IL-10, and IL-35) (Francisco et al., 2009).

PD-1 counters positive signals through the TCR and CD28 receptor by engaging its ligands programmed cell death 1 ligand 1 (PD-L1) and/or PD-L2 (Freeman et al., 2000a)

1.7.2 PD-L1

The gene for PD-L1 (*CD274*) was first identified in 1999 and it was found to be a member of the B7 family of type 1 transmembrane protein receptors. Researchers initially recognised its importance through its inhibitory effects on T-cells by inducing IL-10 (Keir et al., 2008, Dong et al., 1999). The gene for PD-L1 is located on chromosome 9 and comprises 7 exons. The protein is a 290-amino acid protein receptor composed of 2 extracellular domains, IgV- and IgC-like domains; a transmembrane domain and an intracellular domain (Figure 1-4) (Ishida et al., 1992, Freeman et al., 2000b). Interestingly, no function has been attributed to the intracellular domain. PD-L1 is widely expressed by many different cell types and is found on both haematopoietic cells (including T cells, B cells, dendritic cells, and macrophages) and on non-haematopoietic cells, including cell types that upregulate interferons (IFN), interleukins (IL) and anti-inflammatory cytokines through transducer and activation of transcription-1/2 (STAT1/STAT2) (Keir et al., 2008). Pro-inflammatory signals have been shown to induce higher levels of PD-L1 expression (IFNs, TNF, IL-10, IL-27 and some γ-chain cytokines) (Sharpe and Pauken, 2018).

In contrast, PD-L2 expression is much more restricted and this ligand is expressed pre-dominantly by DCs, macrophages and B cells subtypes (Chen et al., 2016). In addition to binding PD-1, PD-L1/PD-L2 also interact with B7 (CD80, CD86) creating negative signals on T cells and dampening antitumor immunity (Butte et al., 2007, Sun et al., 2015). However, the remainder of this chapter will focus on the interaction between PD-1 and PD-L1.

PD-L1 has been extensively researched in the context of immune responses to cancer, and within that context PD-L1 is upregulated in response to two main immune mechanisms, the innate immune resistance and adaptive immune

resistance. In innate immune resistance, tumour cells upregulate PD-L1 on their surface through oncogenic signalling via activation of the PI3K-AKT pathways, following chromosomal alterations or via independent inflammatory signals in the tumour microenvironment (TME) (Marzec et al., 2008, Parsa et al., 2007). For adaptive immune resistance, PD-L1 expression is inducible by response to inflammatory signalling triggered by antitumor responses (Kim et al., 2005, Lee et al., 2005). Several cytokines have been found to be potent inducers of PD-L1 expression on the surface of tumour cells and IFN-y is highly effective (Kim et al., 2005, Lee et al., 2005, Wilke et al., 2011).

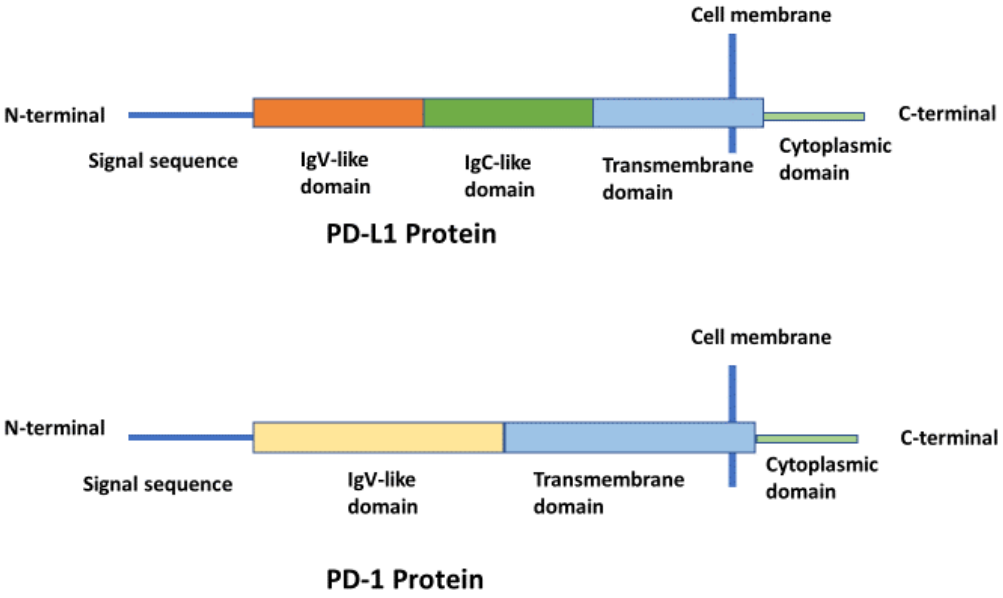


Figure 1-4 The protein structure of PD-L1 and PD-1.

PD-L1 and PD-1 are both transmembrane proteins that interact with each other. PD-L1 mainly contains cytoplasmic domain, transmembrane domain, and two extracellular domains IgV-like and IgC-like. Meanwhile, PD-1 protein only consists of one extracellular domain, transmembrane domain, and cytoplasmic domain.

1.7.3 PD-1/PD-L1 signalling pathway in diabetes

Research into the PD-1/PD-L1 signalling pathway has accelerated over the past 10 years because of its vital role in tumour evasion of the immune system. Within tumour-infiltrating lymphocytes (TILs) PD-1 is expressed in high levels across many types of cancers (melanoma, ovarian, lung and renal carcinomas) and PD-L1 is also expressed and upregulated on many different types of tumour cells (Dong et al., 2002, Zou and Chen, 2008).

Research has demonstrated that the binding of PD-L1 to PD-1 on tumour cells promotes T cell apoptosis, dysfunction, neutralisation, exhaustion and release of IL-10 within the tumour mass for the purposes of causing resistance to cytotoxic T cell (CD8+) mediated tumour cell killing (Zou and Chen, 2008, Sun et al., 2015). These observations were translated into the clinic and early trials with PD-1 and PD-L1 treatment (immune checkpoint inhibitors) showed success in promoting antitumour immune responses. This led to the approval by the US Food and Drug Administration of monoclonal antibodies for therapeutic use in various cancers. However, while proving highly effective in the treatment of cancer, immune-related adverse events were observed in some patients after receiving immunotherapy, including the development of T1D (Day and Hansen, 2016).

Immune-related adverse events following immunotherapy have often involved pancreatic and thyroid tissue (Haanen et al., 2017). A recent literature search found 90 cases where patients were treated with anti-PD-1 or anti-PD-L1 immunotherapy and subsequently developed T1D. Early-onset T1D was observed after 1-2 drug cycles and diabetic ketoacidosis was present in 71% of patients. Islet antibodies were positive in 53% of patients with GADA being the predominant type. Interestingly, the dominant HLA serotypes were DR4 (most

dominant), DR3, DR9 and A2 (de Filette et al., 2019). This clearly indicated that the PD-1/PD-L1 signalling pathway could play a role in the development of T1D.

Wang et al. (2008) investigated the protective potential of PD-L1 in autoimmune diabetes by transgenically overexpressing PD-L1 in pancreatic β-cells in NOD mice. They observed a significant decrease in the severity of insulitis and disease onset was delayed. The overall incidence of diabetes was markedly decreased compared with controls. Moreover, NOD mice that received lymphocytes from transgenic mice became diabetic at a slower rate than their control counterparts. This suggested a protective role for PD-L1 in the development of diabetes in NOD mice (Wang et al., 2008) .

1.7.4 Forms of PD-L1 expression

PD-L1 can be expressed on the surface of tumour cells, immune cells and other cells in the tumour microenvironment but it can also be found in extracellular forms. Human blood contains several forms of soluble or extracellular PD-L1, including packaging within exosomes and microvesicles. Studies have indicated that expression of PD-L1 in exosomes can affect the tumour microenvironment, and directly and systemically inhibit an antitumour immune response (Chen et al., 2018, Daassi et al., 2020).

1.8 Exosomes and Extracellular Vesicles

As explained in earlier sections, β-cells are specialised cells that secrete granules carrying hormones that regulate metabolic activities. They also, like all cells, secrete various other types of membrane vesicles known as extracellular vesicles (EVs).). The release of EVs into the extracellular space is a conserved process and can be evolutionary traced in bacteria, humans and plants (Schorey et al., 2015, Deatherage and Cookson, 2012, Robinson et al., 2016). Researchers initially relegated the release of EVs to ‘waste removal processes’

and believed cells were exporting waste products through these vesicles (Johnstone et al., 1987). However, decades of extensive research has shown that EVs can act as vital messengers between cells, carrying nucleic acids, lipids, proteins and other molecules to regulate normal homeostatic processes (Figure 1-6). Extracellular vesicles carry coding and non-coding RNA, miRNAs and DNA sequences (Kahlert et al., 2014) which can potentially influence gene expression in recipient cells. In instances of disease or pathology, EVs can change molecular character and carry biomarkers indicative of particular pathological alteration (Colombo et al., 2014, Cicero et al., 2015, Yáñez-Mó et al., 2015).

Extracellular vesicles are a population of heterogenous vesicles of differing sizes, functions and properties (Table 1-2). Based on research to date, EVs have been categorised into two main groups: microvesicles and exosomes (Table 1-3). The label ‘exosome’ was originally used to describe vesicles observed to leave cultured cells carrying 5´nucleotidase activity (Trams et al., 1981), before being used to describe vesicles between 30-100 nm released from reticulocytes during differentiation (Johnstone et al., 1987). Generally formed by the inward budding of the endosomal membrane during maturation of multi-vesicular endosomes (MVEs), exosomes are secreted into the extracellular space after fusion of MVEs with the cell surface (Figure 1-5) (Harding et al., 1984, Pan et al., 1985).

Microvesicles were initially recognised for their role in blood coagulation but more recently have been found to play vital roles in cell-to-cell communication (Satta et al., 1994, Al-Nedawi et al., 2008). Microvesicles are generally described as being within the size range of 50-1000 nm in diameter and form

after the outward budding and fission of the plasma membrane occurs, followed by release into the extracellular space (Figure 1-5) (Tricarico et al., 2017).

	Exosomes	**Microvesicles**
Origin	Endosome	Plasma membrane
Size	50-150 nm	50-500 nm (up to 1000nm)
Other names (according to their origin, size and morphology	• Prostasomes • Tolerosomes • Dexosomes • Nanovesicles • Exosome-like vesicles and others	• Microparticles • Blebbing vesicles • Shedding vesicles • Oncosomes • ARRMs • Migrasomes • Neurospheres • Apoptotic bodies

Table 1-2 The origin and size of microvesicles and exosomes.

Extracellular vesicles comprise a heterogeneous population of membrane vesicles of various origins. Their size may vary (50 - 500 nm but can reach 1–10 μm). Extracellular vesicles have been named based on their origin (cell type), size, morphology and cargo content but are now classified into two distinct classes: exosomes and microvesicles.

Exosomes				
Membrane organisers Tetraspanins: CD9, CD81, CD63, TSPAN8, CD151, CD37, CD53, Flotilin 1 and 2	**Lipids** Phosphatidylserine, cholesterol, ceramide and other sphingolipids	**Cell adhesion** E.g. integrin, lactadherin, ICAM	**Intracellular trafficking** E.g. RAB, GTPases, annexins	**Cell-type-specific proteins** E.g. MHC-I, MHC-II, APP, PMEL, TCR, FasL, CXCR4, HSPG, CD86, PrP, TFR, WNT
Enzymes Peroxidases, pyruvate kinase, enolase, GAPDH	**Signal transduction** Protein kinases, β-catenin, 14-3-3, G proteins	**Biogenesis factors** ALIX, TSG101, syntenin, ubiquitin, clathrin, VPS32, VPS4	**Chaperones** HSP70, HSP90	**Nucleic acids** microRNA and other non-coding RNAs, mRNA, DNA (and histones)

Microvesicles				
Membrane organisers Tetraspanins: CD9, CD81, CD82	**Lipids** Phosphatidylserine, phosphatidylethanolamine, sphingolipids	**ECM adhesion** E.g. integrin, PECAM1, fibronectin	**Intracellular trafficking** E.g. RAB, GTPases, annexins	**Cell-type-specific proteins** E.g. MHC-I, LFA1, CD14
Cytoplasmic material Tau, TDP43, GAPDH	**Signalling molecules** For example, ARF6, RAB11, ROCK	**Biogenesis factors** ALIX, TSG101, ERK, PLD, VPS4	**Chaperones** HSP70, HSP90	**Nucleic acids** microRNA and other non-coding RNAs, mRNA, DNA (and histones)

Table 1-3 Main features of extracellular vesicles.

Study of extracellular vesicle composition revealed that they can carry various cargoes, including proteins, lipids and nucleic acids, and this content can vary widely between cells and conditions. The particular composition will directly affect the fate and function of extracellular vesicles, strengthening the importance of selective cargo-sorting mechanisms. Of note, depending on the cell type, extracellular vesicles will display a set of cell-type-specific proteins that account for their specific fates and functions. Despite a different mode of biogenesis, exosomes and microvesicles display a similar appearance,

overlapping size and often common composition that make it difficult to ascertain their origin once isolated from the extracellular medium or from biological fluids. ALIX, ALG-2 interacting protein X; APP, amyloid precursor protein; ARF6, ADP-ribosylation factor 6; ARMMs, arrestin-domain-containing protein 1-mediated microvesicles; CXCR4, CXC-chemokine receptor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP70, heat shock 70 kDa protein; HSPG, heparan sulfate proteoglycan; ICAM, intercellular adhesion molecule; LBPA, lyso-bis-phosphatidyl acid; LFA1, lymphocyte function-associated antigen 1; MHC, major histocompatibility complex; PECAM1, platelet endothelial cell adhesion molecule; PLD, phospholipase D; PrP, prion protein; ROCK, RHO-associated protein kinase; TCR, T cell receptor; TDP43, TAR DNA-binding protein 43; TFR, transferrin receptor; TSG101, tumour susceptibility gene 101 protein; TSPAN, tetraspanin; VPS, vacuolar protein sorting-associated protein. Table inspired from van Niel et al. (2018).

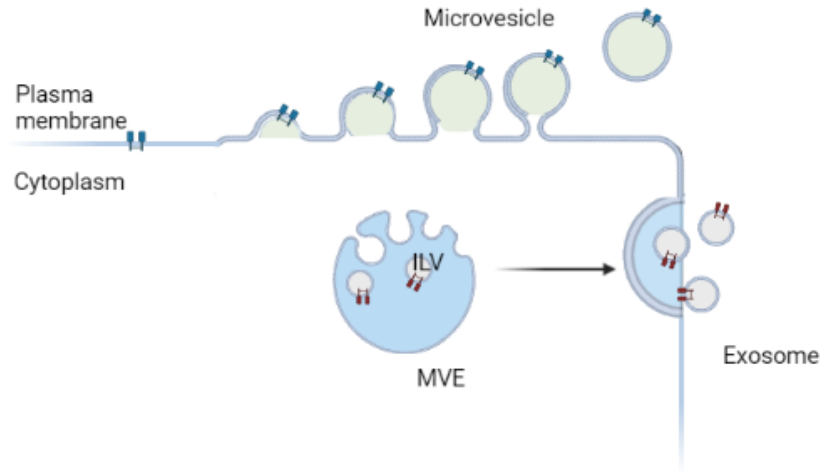


Figure 1-5 Release of exosomes and microvesicles from the cell.

Extracellular vesicles are formed either by budding of the plasma membrane, in which case they are referred to as microvesicles, or as intraluminal vesicles (ILVs) within the lumen of multivesicular endosomes (MVEs). MVEs fuse with the plasma membrane to release ILVs that are then called exosomes. Figure created in Biorender.

Commonly used protocols for recovering extracellular vesicles from liquid biopsy or cell culture media lead to the collection of a heterogenous population of vesicles (Willms et al., 2016). Despite the different sites of biogenesis of exosomes and microvesicles, there are overlapping intracellular mechanisms and sorting machineries for both vesicles, which can encumber methods to distinguish the two subpopulations of vesicles (Colombo et al., 2014). This population will also contain other cellular bodies such as apoptotic bodies, migrasomes, and arrestin-domain-containing protein 1- mediated microvesicles (Nabhan et al., 2012). The latter buds directly from the plasma membrane similarly to viruses and relies on endosomal sorting complex required for transport (ESCRT) proteins. Due to overlapping sizes, non-distinct morphology and differences in composition, creating a more precise nomenclature for extracellular vesicles is difficult (Gould and Raposo, 2013, Kowal et al., 2016).

The cargo found within vesicles is cell-type specific and influenced by the biological state of the donor cell, the signalling that modulates the production of cargo, and the routes of biogenesis (Minciacchi et al., 2015). For example, cargo such as the expression of the major histocompatibility complex (MHC) class II (Ostrowski et al., 2010) promotes MVE formation leading to release of exosomes.

Cargo typically found in exosomes has moved through the Golgi apparatus to the endosomes, or been internalised from the plasma membrane, before sorting to ILVs during endosome maturation (Klumperman and Raposo, 2014). Within the lumen of endosomes during the maturation phases into MVEs, exosomes are generated as ILVs. This process is underpinned by sorting machineries that separate cargo on microdomains of MVEs followed by inward budding and

fission of small membrane vesicles containing cytosolic components. The ESCRT machinery drives the formation of MVEs and ILVs (Hurley, 2008), and acts in a phased manner.

Additionally, exosomes can also form in an ESCRT-independent manner (Stuffers et al., 2009). Proteins of the tetraspanin family have been shown to regulate ESCRT-independent endosomal sorting (ex. CD63). (Theos et al., 2006). Tetraspanins CD81, CD82 and CD9 are also involved in the sorting of various cargo into exosomes (Buschow et al., 2009). The movement of cytosolic proteins into ILVs can occur through sorting with proteins such as heat shock 70kDa protein (HSP70), a protein found in exosomes released from most cell types (Théry et al., 2001, Géminard et al., 2004).

The biogenesis of microvesicles has also been shown to involve lipid components, protein complexes, Ca^{2+} levels, and Ca^{2+} dependent mechanisms such as aminophospholipid translocases, scramblases and calpain modifications of membrane phospholipids. (Minciacchi et al., 2015). An important lipid component is cholesterol, which is found abundantly in microvesicles (Del Conde et al., 2005).

Once released into the extracellular space, EVs are transported to their recipient cells and deliver their cargo to effect functional responses and promote phenotypic changes. This process of intercellular communication requires docking at the plasma membrane, which results in activation of surface receptors and internalisation of the vesicle (endocytosis), or the physical fusion with target cells (Mulcahy et al., 2014), however, the exact mechanisms governing these processes have yet to be elucidated. The directional flow of EVs to their targets may be guided by the proteins enriched on the surface of

the EVs as well as by receptors present on the surface of the recipient cells (Denzer et al., 2000). The interaction between the EVs and the recipient cells plasma membrane can be mediated by several factors including tetraspanins, integrins, lipids, lectins, heparan sulfate proteoglycans and extracellular matrix components (Sung et al., 2015).

Once docked, EVs can cause functional changes to their recipient cells, and examples of this include exosomes from B cells and dendritic cells presenting antigens to T cells resulting in an induction of antigenic response. Cargo can also be processed in the endocytic compartment of the recipient cells, similar to antigens, and then used in antigen presentation (Raposo et al., 1996). Transfer of lipids such as cholesterol has also been shown to occur in EVs (Record et al., 2014) and cargo can be directed to lysosomes for degradation of proteins and lipids, potentially providing a source of metabolites for the recipient cells (Zhao et al., 2016).

The large volume of research on EVs has led to the potential use of EVs and microvesicles as biomarkers of health and disease states. By understanding the cellular origins of EVs or microvesicles, protein signatures can be mapped to physiological changes and disease progression. EVs also harness the potential for novel immunotherapies, with studies currently underway for treating lung cancer where EVs secreted by the immune cells are tested for their ability to stimulate the immune system (Lener et al., 2015).

1.8.1 The role of EVs in the pathology of diabetes

Under certain conditions, immune cells can communicate with other cells through EV release and uptake. Studies on the presence of EVs in T1D have found that pancreatic islet infiltrated lymphocytes can secret EVs capable of transferring miRNAs to β-cells and trigger the activation of apoptotic pathways

(Guay et al., 2019). β-cell EV release can also affect immune cell functions through either direct lymphocyte activation or EV-carrying autoantigen release and subsequent uptake by APCs (Vomund et al., 2015, Cianciaruso et al., 2017, Guay et al., 2019). Interestingly, vesicles have also been observed to transfer insulin to tissue-resident APCs (Vomund et al., 2015) and a recent study discovered that human islet-derived EVs are able to specifically activate memory T cells derived from T1D patients but not those from non-diabetic control subjects (Rutman et al., 2018). All together, these studies have demonstrated active communication between β-cells and the immune system through the use of EVs.

EVs are known to play a role in the antiviral responses against a breath of viruses. Interestingly, T-cell derived EVs containing TCR/CD3 complexes have been associated with antiviral immune responses in non-diabetic models, and feedback mechanisms have been observed by which T cell-derived EVs enhance the activity of APCs in response to infections (Zhou et al., 2020). Conceivably, this activity may occur during enteroviral infection of pancreatic islets, but research on this topic has not been undertaken. Indeed, EVs are even known to contain viruses themselves, as EV biogenesis is hijacked by enteroviruses to aid intercellular transmission and immune evasion (Netanyah et al., 2020). However, research on persistent enteroviral infection altering pancreatic cell function and EV biogenesis is needed to explore this topic.

The role of EVs in T1D is gradually being uncovered, but given the function of EVs to carry lipids, proteins and nucleic acids, their presence is likely to be significant. For example, studies with NOD mice have demonstrated that EVs from β-like cells can induce secretion of inflammatory molecules such as IL-6, IFN-γ, TNF-α, MCP-1, and IL-10 (Sheng et al., 2011). EVs may become useful

biomarkers of disease progression or immune infiltration into the islet, and their communication networks may be exploited for clinical intervention to send immunomodulatory messages to the immune cells.

1.9 Project Aims

The overarching broad aim of this study was to determine if immunomodulatory proteins such as PD-L1 are involved in the development of T1D. Given the evidence that PD-L1 may play a vital role in controlling the immune system through interactions with PD-1, the first step is to determine if PD-L1 and other immunomodulatory proteins are present in the pancreatic tissue of subjects with T1D. Mechanisms of transfer of PD-L1 were then investigated by studying EV release from clonal β-like cells.

This study also originally aimed to determine if enteroviruses could affect the composition and release of EVs from pancreatic cells, or indeed if enteroviruses were hijacking EVs as a form of intercellular transport during persistent infection. However, the COVID-19 pandemic significantly affected these aims and revised aims were constructed to analyse the methylation signature of persistent infections on pancreatic cells. This is considered important because of the known links between persistent enteroviral infection and T1D in children. Understanding how the virus can manipulate the host cell will provide a better understanding of how viruses can create an environment conducive to persistent infection and whether this influences β-cell physiology.

When lockdown was enforced across the UK to control the rising number of COVID-19 infections, I also utilized this time to analyse publicly available online transcriptomics data to investigate the relationship between pancreatic ductal adenocarcinoma (PDAC) and diabetes. This was undertaken as a skills development study but is also considered important because there remain unanswered questions as to whether T3cDM is a unique form of diabetes or, rather, a form of T2D.

To briefly summarise the aims:

(1) To utilise human pancreas samples to investigate and compare the expression of PD-L1 and other immunomodulatory proteins in donors with T1D and those with no diabetes.

(2) To develop effective methods of isolation of EVs from β-cell lines and to characterise the expression of EVs and immunomodulatory markers.

(3) To determine if concomitant diabetes alters the gene expression profiles of bulk tumour samples from patients with PDAC, and investigate whether diabetes is associated with a certain PDAC tumour subtype.

(4) To establish a persistent and acute enteroviral infection in pancreatic cells and determine if cellular methylation signatures vary between the two states of infection.

Chapter 2

2 Evidence of immune checkpoint expression in Type 1 diabetes

2.1 Introduction

The immune system has evolved to eliminate threats from foreign organisms, however, as explain in **Chapter one** it must do so while preventing harmful inflammatory responses that would lead to the destruction of host tissue, This task is achieved through multiple checks and balances on immune responses that function during lymphocyte development in central lymphoid organs (central tolerance) and the periphery (peripheral tolerance) (Table 2-1) (Sharpe and Pauken, 2018, Francisco et al., 2010).

Central tolerance	Peripheral tolerance
Mechanisms of tolerance that occur in the central lymphoid organs (thymus for T cells, bone marrow for B cells). Mechanisms include negative selection (for both T cells and B cells), receptor editing (for B cells) and lineage deviation (for T cells)	Broadly refers to a series of mechanisms used by the body to limit the activation of self-reactive T cells and B cells to prevent these cells form targeting and destroying self-tissues.

Table 2-1 Summary of central and peripheral tolerance.

For T cells, regulatory mechanisms are activated alongside the initial antigen-mediated activation, which involves T cell receptor (TCR) engagement with an MHC-peptide and positive co-stimulatory signals (ex. CD28 on T cells and CD80 on antigen-presenting cells (APC)). Initial activation processes induce negative regulators to counteract the activation programme. One of the first negative regulators to be induced is the cytotoxic T lymphocyte antigen 4 (CTLA4) and directly competes with CD28 for CD80/CD86. PD-1 is also

expressed in the initial activation process and counters positive signals through the TCR and CD28 by engaging its ligands PD-L1 and PD-L2 (Figure 2-1). These receptors are referred to as ‘coinhibitory’ and act as breakers for the immune system response. They serve as immune checkpoints that T cells must pass to attain full functionality.

Inhibitory signals have evolved to allow the immune system to maintain balance. Disrupting the PD-1/PD-L1 pathway can have profound impacts on the host physiology, as seen in mice genetically deficient in *Pdcd1* (gene encoding PD-1) who develop accelerated autoimmunity (Nishimura et al., 2001, Nishimura et al., 1999, Lucas et al., 2008). Moreover, NOD mice genetically deficient in *CD274* (gene encoding PD-L1) will develop T1D (Wang et al., 2005). Conversely, sustained expression of PD-1 or PD-L1 is common during chronic infections and cancer because promoting negative regulators of T cell function can support both viruses and tumours to escape immune clearance (Pauken and Wherry, 2015, Barber et al., 2006, Iwai et al., 2002, Hirano et al., 2005).

In clinical settings, treatment with early PD-1 and PD-L1 pathway inhibitors demonstrated success in promoting anti-tumour immune responses and prolonged patient survival. This led the approval of several anti-PD-1/PD-L1 immunotherapies to treat various cancers (Page et al., 2014, Topalian et al., 2015). However, multiple cases of immune-related adverse events (IRAEs) were recorded after administration of PD-1/PD-L1, and there have been multiple instances of individuals developing T1D (Barroso-Sousa et al., 2018).

Coupled with rodent studies (Ansari et al., 2003, El Khatib et al., 2015, Li et al., 2015) these findings suggest that PD-1/PD-L1 may play an important role in

regulating the immune tolerance towards insulin-producing β-cells. T1D is known to be accompanied with high levels of inflammation and insulitis (Morgan et al., 2014). It could be postulated that anti-PD-1/PD-L1 immunotherapy may disrupt the binding of PD-1 to its ligand PD-L1 on endocrine cells, thus allowing the immune cells to destroy the islets and trigger diabetes. As we know that individuals with T1D are often genetically susceptible to the disease, it may be the case that individuals who developed T1D after immunotherapy were genetically susceptible to T1D but the regulatory actions of the PD-1/PD-L1 pathway in some way prevented the progression of T1D. To determine if this is the case, it is incredibly important to examine PD-L1 or PD-1 is expression in the human pancreas, and aim to understand its role in the development of diabetes at the molecular level.

Studies across several cancer types have demonstrated that several pro-inflammatory signals can induce higher levels of PD-L1 expression. These include pro-inflammatory stimuli from the interferon family (IFN-α, IFN-β, IFN-γ, and IFN-λ) (Figure 2-2) as well as interleukins (IL-1β, IL-6, IL-10, IL-12) and TNF-α (Sun et al., 2018). Across several cancer types, these stimuli have been found to activate the JAK-STAT-IRF1 signalling pathway (for example, in melanoma cells) (Garcia-Diaz et al., 2017), but other pathways such as the NF-κB-stimulated lipopolysaccharide pathway in macrophages can also mediate expression of PD-L1 (Loke and Allison, 2003). The process by which interferons can regulate PD-L1 expression has contributed to the concept of ‘adaptive resistance’ in tumours, which suggests that PD-L1 expression is further increased within the tumour microenvironment as a result of pro-inflammatory cytokines released by infiltrating T cells. This in turn promotes immunosuppression.

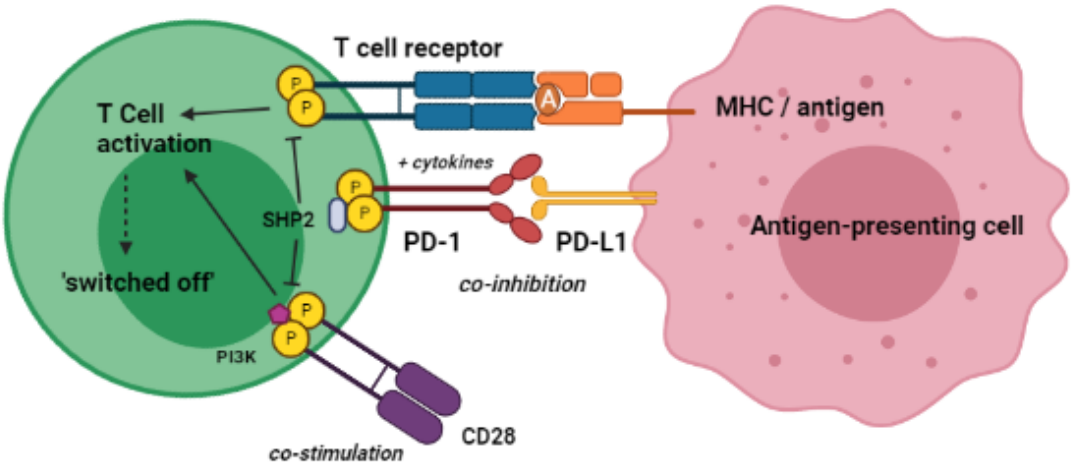


Figure 2-1 Diagram showing the PD-1/PD-L1 immune checkpoint pathway. Antigens are presented to the T cell receptor (TCR) through MHC molecules. Antigen/ MHC binding to the TCR triggers a signalling cascade which activates the T cell, this occurs in conjunction with a combination of co-stimulatory molecules and cytokines that programme the intensity of the activation. PD-1 is an inhibitory receptor induced upon T cell activation and engages with its ligand PD-L1 on the antigen-presenting cell. Engagement of PD-1 with PD-L1 leads to phosphorylation of tyrosine residues on the immunoreceptor tyrosine-based switch motif (ITSM). This triggers recruitment of Src homology phosphatase 2 (SHP2) to the ITSM, leading to inhibition of TCR signalling and CD28-mediated PI3K activation. This results in downmodulation of T cell activity and function.

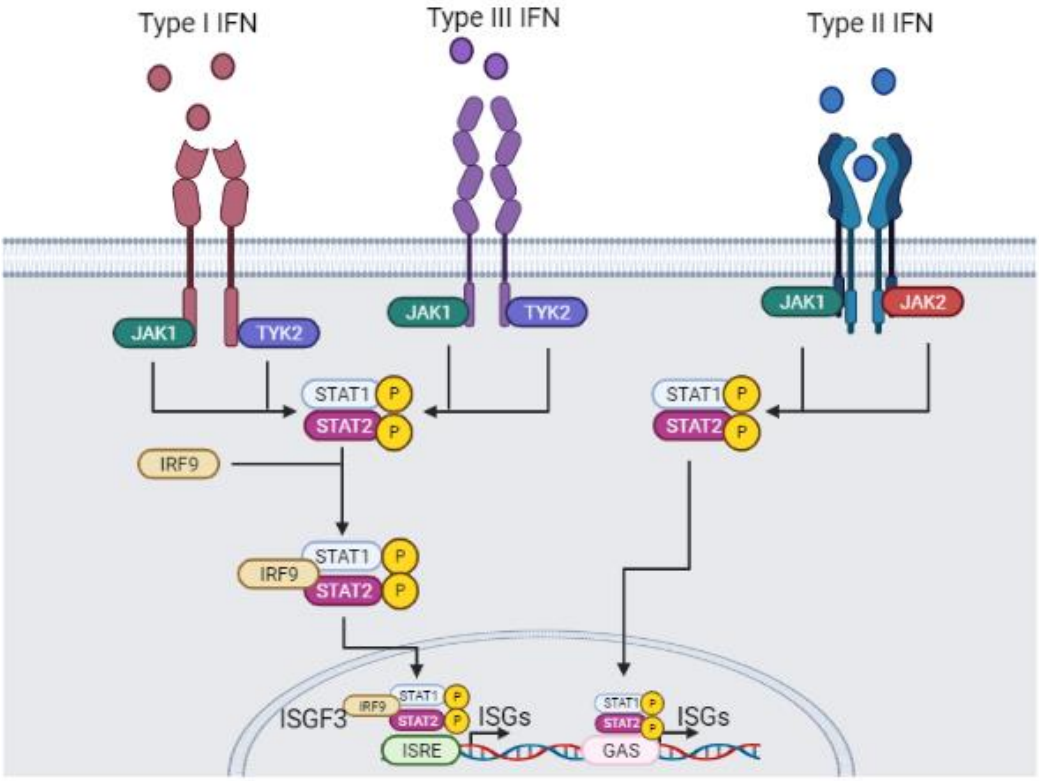


Figure 2-2 Schematic diagram showing interferon signalling

Type I, II and III interferons have specific receptors, which are associated with specific Janus kinases, essential for mediating downstream signalling pathways. The type I IFN (IFN-α, -β) and type III IFN (IFN-λ) receptors are associated with the Janus kinases JAK1 and TYK2. These auto-phosphorylate upon ligand-receptor binding and mediate STAT1 and STAT2 phosphorylation and subsequent dimerization. These then associate with IRF9 to form the ISGF3 complex, which can mediate DNA binding at ISRE sites. The type II IFN (IFN-γ) receptor is associated with the Janus kinases JAK1 and JAK2. These auto-phosphorylate upon ligand-receptor binding and mediate STAT1 dimerization. STAT1 homodimers bind GAS on DNA, and mediate transcription of a specific subset of ISGs. Diagram created using Biorender.

In the context of T1D, the most intriguing signalling pathways to investigate would be the IFNs (IFN-α, IFN-β and IFN-γ) since they are known to play a role in shaping the T1D landscape. IFN-α transcriptional signatures have been observed during the development of autoimmunity in genetically at-risk children (Ferreira et al., 2014) and are expressed in islets of individuals with T1D (Huang et al., 1995). Transcriptome analysis reveals a type I IFN signature in the peripheral blood of patients prior to the development of autoantibodies (Kallionpää et al., 2014, Ferreira et al., 2014). Moreover, development of T1D was reported in some patients receiving type I IFN therapy for various conditions including Hepatitis C and cancer (Guerci et al., 1994, Nakamura et al., 2011, Oka et al., 2011). Other studies have demonstrated upregulation of IFN stimulated genes STAT1 and HLA-ABC in T1D at the mRNA and protein levels (Lundberg et al., 2016, Richardson et al., 2016, Foulis et al., 1987, Pujol–Borrell et al., 1987).

After binding to its receptor, IFN-α promotes phosphorylation of two tyrosine kinases, JAK1 and TYK2, which then trigger the downstream signalling cascade (Figure 2-2). Interestingly, drugs that chemically target JAK1/JAK2 have been shown to prevent autoimmune diabetes in NOD mice (Trivedi et al., 2017) and polymorphisms associated with decreased TYK2 function are protective against human T1D (Marroqui et al., 2015). Overall, this demonstrates, in theory, that the signalling pathways that promote expression of PD-L1 on cells are active in the T1D landscape.

The main aim for this Chapter was to determine if expression of PD-L1 and PD-1 was present in the pancreas of individuals with T1D. This is important because it would help to explain why some individuals develop autoimmune disease after receiving anti-PD-L1 or anti-PD-1 immunotherapy. This Chapter

would be the first to explore expression of PD-L1 in the human pancreas and record observations across whole tissue sections because islets can display incredible levels of heterogeneity across the pancreas (Dybala and Hara, 2019). To complete my analysis, I will access three different pancreatic tissue collections. As research from the islet biology team at the University of Exeter continues to unravel early life differences in T1D at the molecular level (Leete et al., 2020), I will determine if age or duration of disease are influencing factors in the expression of PD-L1/PD-1.

Further aims of this Chapter include investigating any additional immune checkpoints or proteins involved in the dialogue between immune cells and β-cells. This dialogue is important to understand because researchers have spent decades debating the level of 'blame' on the immune system for mediating T1D, with some arguing that dysfunction of the β-cell provokes an immune attack. Looking beyond the original trigger of disease, however, the dialogue continues but there is limited evidence demonstrating that β-cells attempt to stop the immune attack after its ascent. Finding evidence of β-cells expressing molecules that could slow an immune assault would support the clinical observation that the pathogenesis and timing of symptoms is highly variable across the population (Ilonen et al., 2019). At the time of T1D onset, which occurs with the presentation of symptoms, individuals have different levels of β-cell loss, detected by measuring serum levels of C-peptide (a by-product of insulin synthesis), which contributes to heterogeneity of T1DM. Understanding the immunological factors that contribute to these differences is important to stratify different progression phenotypes and could inform future phenotype-dependent immunotherapies.

2.2 Methods

2.2.1 Tissue

Formalin-fixed, paraffin embedded human pancreatic sections were selected based on age from the Exeter Archival Diabetes Biobank (EADB; http://foulis.vub.ac.be/). Additional adult human sections were obtained from the DiViD biopsy study of living donors with recent-onset T1D (Krogvold et al., 2014). Tables 2-2 and 2-3 expand on subject characteristics. In summary, 6 non-diabetic and 12 T1D subjects were studies. All samples were studied with appropriate ethical approval. EADB samples were used with ethical permission from the West of Scotland Research Ethics Committee ((ref: 20/WS/0074; IRAS project ID: 283620). In the case of the DiViD study, participants provided written informed consent (Krogvold et al., 2014, Krogvold et al., 2015a).

2.2.2 Immunohistochemistry

Formalin-fixed, paraffin embedded pancreatic sections were dewaxed, rehydrated and subjected to heat-induced epitope retrieval (100°C for 10 min) using Citrate buffer (10 mM, pH6), Universal HIER Antigen Retrieval Reagent (Abcam, UK) (when followed by probing with PD-L1 antibodies), or Citrate buffer followed by a second antigen retrieval with 1mM EDTA (pH8) (when followed by probing with CD3+ and CD8+ antibodies).Tissue sections were then blocked with 5% normal goat serum before incubation with primary antibodies (Table 2-4). TBS was used to wash the tissue sections three times and peroxidase black was applied to inhibit the activity of endogenous peroxidase activity. Secondary antibodies (Table 2-5) were part of the Dako (UK REAL™ EnVision™ Detection System (Cat #K5007) and were capable of recognising mouse and rabbit primary antibodies. The system uses a dextran backbone conjugated to multiple horse-radish peroxidase (HRP) molecules to amplify the signal. The HRP reacted with 3,3'-diaminobenzidine (DAB) + DAKO Real

substrate buffer to locate the proteins reacting with the antibodies (brown colour in images). To identify the nuclei of cells, haematoxylin was applied to the tissue sections before washing with H_20. Copper sulphate (2%) in saline solution (0.9%) was applied to intensify the DAB stain, and then the tissues were dehydrated in stepwise increasing ethanol concentrations. Mounting and fixing was done using DPX before viewing on a brightfield microscope (Nikon 50i microscope).

2.2.3 Immunofluorescence

Samples were dewaxed, rehydrated and subjected to heat-induced epitope retrieval in Universal Buffer (Abcam, UK) then probed with antibodies (Table 2-4). The relevant antigen-antibody complexes were detected using secondary antibodies conjugated with fluorescent dyes (Alexa Fluor™ anti-mouse 555, anti-rabbit TSA 488, anti-guinea pig 647) (Invitrogen, Paisley, UK) (Table 2-5). Cell nuclei were stained with DAPI. After mounting, images were captured with a Leica AF6000 microscope (Leica, Milton Keynes, UK) and processed using the standard LASX Leica software platform.

For quantification studies with PD-L1 and HLA-E, randomly selected insulin-containing islets (ICIs) from individuals with or without diabetes were imaged, in addition to insulin-deficient islets (IDIs) from individuals with diabetes. Thirty ICIs were analysed from 6 separate individuals (5 islets per individual), 20 and 25 IDIs (HLA-E and PD-L1 analysis respectively) were analysed from 4 and 5 separate individuals (5 islets per individual) and 30 ICIs were analysed from 6 separate control individuals (5 islets per individual). The mean fluorescence intensity (MFI) arising from detection of PD-L1 and HLA-E was measured using LASX Leica quantification software.

2.2.4 Statistical Analysis

A significant difference between experimental conditions was assessed by two-way ANOVA followed by Bonferroni correction for multiple comparisons as indicated using the GraphPad Prism program version 6.0 (www.graphpad.com). Results with $p \leq 0.05$ were considered statistically significant.

Sample	Identity	Age (years)	Sex	Disease Duration
DiViD 1	T1D	25	Female	4 weeks
DiViD 2	T1D	24	Male	3 weeks
DiViD 3	T1D	34	Female	9 weeks
DiViD 4	T1D	31	Male	5 weeks
DiViD 5	T1D	24	Female	5 weeks
DiViD 6	T1D	35	Male	5 weeks
6228-04 PB	T1D	13	Male	0
6099-01 PB	ND	14.2	Male	-
6047-02 PH	ND	7.8	Male	-
6095-04 PT	ND	40	Male	-
6024-08 PT	ND	21	Male	-
6160-03 PH	ND	22.1	Male	-

Table 2-2 Details of nPOD and DiViD samples utilised in the study

(Krogvold et al., 2014, Krogvold et al., 2015a). The first 4 digits of the sample number are the patient ID, the second 2 numbers indicate the block number. PH = Pan head, PB = Pan body and PT = Pan tail. T1D = Type 1 diabetes, ND = Non-diabetic control

Sample	Identity	Age	Duration of T1D
SC115	T1D	1.3	At onset
SC41	T1D	4	3 weeks
SC119	T1D	4	2 weeks
242/89	NDC	3	N/A
21/89	NDC	4	N/A
184/90	NDC	5	N/A
E261	T1D	18	3 weeks
E124B	T1D	17	Recent
E556	T1D	18	4 months
146/66	NDC	18	N/A
191/67	NDC	25	N/A
333/66	NDC	16	N/A
E560	T1D	42	18 months
12495	Neonate Paediatric	1 week	N/A
12424	Neonate Paediatric	3 weeks	N/A
18/88	NDC	68	N/A
113/66	NDC	80	N/A
7121A_75	Tonsil	N/A	-

Table 2-3 Details of the EADB cohort samples that were utilised in this study.

T1D = Type 1 diabetes, NDC = Non-diabetic control

Antigen	Catalogue # and company	Raised in	Dilution	Incubation Time
PD-L1	ab205921 Abcam	Rabbit	1/100	O/N then 1 hr at RT
Insulin	A0564 Dako	Guinea-Pig	1/363	1 hr
Insulin	IR00261-2 Agilent	Guinea-Pig	1/5	1 hr
Glucagon	ab10988 Abcam	Mouse	1/2000	1 hr
PD-1 [EPR4877(2)]	ab137132 Abcam	Rabbit	1/200	O/N
PD-1	ab234444 Abcam	Mouse	1/50	O/N
TIGIT Clone TG1 Recombinant peptide from extracellular domain of human TIGIT Membranous	DIA-TG1-M Dianova	Mouse	1/33	O/N then 1 hr at room temperature
HLA-E (MEM-E102)	ab2216 Abcam	Mouse	1/1500	1 hr
IRF1	Cell Signalling Ca8478	Rabbit	1/100	O/N then 1 hr at RT

Table 2-4 Primary antibodies used in this chapter

Reactive against	Conjugated to	Company	Cat#	Raised in	Dilution	Incubation time	App.
Rabbit IgG	488	Life Tech	#A11034	Goat	1/400	1	IF
Rabbit IgG	555	Life Tech	#21429	Goat	1/400	1	IF
Mouse IgG	488	Life Tech	#A32723	Goat	1/400	1	IF
Mouse IgG	555	Abcam	ab150114	Goat	1/400	1	IF
Guinea-pig IgG	555	Life Tech	A21435	Goat	1/400	1	IF
Guinea-pig IgG	647	Life Tech	A21450	Goat	1/400	1	IF
Goat IgG	488	Life Tech	A11055	Donkey	1/400	1	IF
Rabbit/Mouse IgG	HRP	Agilent	K5007	Goat	N/A	0.5	IHC

Table 2-5 Secondary antibodies used in these studies.

All secondary antibodies are incubated at room temperature. (App. = application)

2.3 Results

2.3.1 PD-L1 is expressed in the β-cells of individuals with Type 1 diabetes

Initial experiments were conducted in tonsil tissue to compare suitable antigen-retrieval methods for immunohistochemistry. Two different buffers, 10mM Citrate (Tris-sodium citrate, H_2O) buffer (pH 6.0) and Universal buffer (patented solution) (Abcam, UK), were compared for efficacy during the heat-induced epitope retrieval (HIER) step (Figure 2-3). The Universal buffer proved to be a more effective treatment for retrieving antigens as PD-L1 was strongly detected in a subset of epithelial cells within the tonsil tissue. Therefore, IHC and IF experiments with the PD-L1 antibody in Chapter 2 were conducted with a HIER step that used Universal Buffer. As expected, PD-L1 was not detected in the lymph nodule.

Once the conditions for testing were optimised, an immunofluorescence approach was employed to explore PD-L1 expression in human pancreatic tissue (EADB collection). Pancreatic tissue sections from a subject with acute autoimmune pancreatitis were used as a reference case representing high inflammation with predicted high numbers of CD8+ T cells (Gupta et al., 2019). PD-L1 was co-stained with insulin and glucagon to identify which endocrine cell type expressed the protein. PD-L1 expression was observed in the β-cells of some islets and individual acinar cells within the exocrine tissue (Figure 2-4A).

PD-L1 expression was then assessed in the islets of donors with T1D (n=8) and aged-matched donors without diabetes (n=6). Five insulin-containing islets (ICIs) were randomly selected in each of the different donor types for quantification analysis, as well as five insulin-deficient islets (IDIs). Positive PD-L1 immunostaining was present in both young (sc41; Figure 2-4B) and adult subjects (E560), but absent (or very weak, at best) in donors without diabetes

(Figure 2-4B, lower panel). A strong association was found between PD-L1 expression and insulin-containing β-cells, with occasional occurrence in the α-cells. Interestingly, PD-L1 expression was not observed in insulin-deficient islets (IDIs) of the subjects with T1D (Figure 2-4B & C). PD-L1 expression was significantly higher ($p<0.05$), as calculated by mean fluorescence intensity (MFI), in the ICIs of T1D individuals compared to the IDIs and non-diabetic controls (Figure 2-4C).

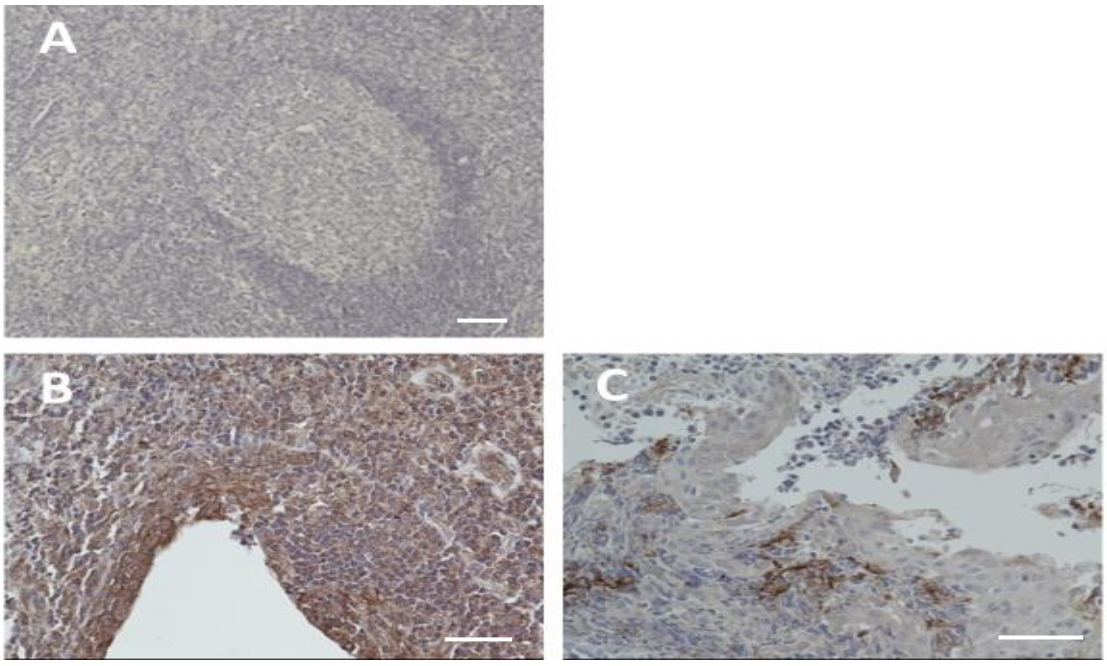
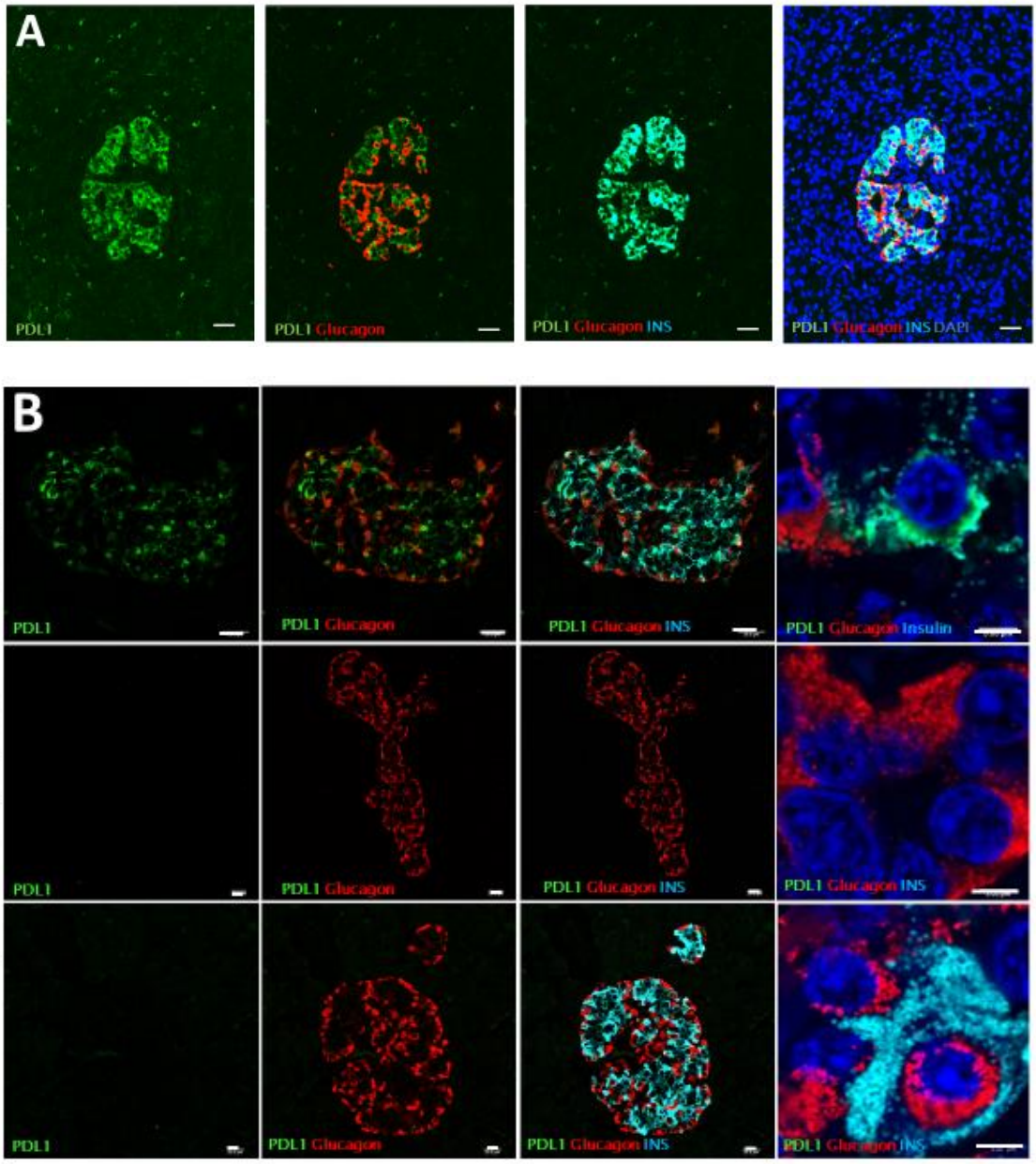


Figure 2-3 PD-L1 is expressed in human tonsil tissue. PD-L1 positive cells, shown in brown (DAB staining), were located in the tonsil epithelium in the sections treated with Universal buffer HIER (Modern Pathology (2018) 31:1630–1644 https://doi.org/10.1038/s41379-018-0071-1). Optimisation of the HIER method with the PD-L1 antibody was conducted using (A) citrate buffer and (B)(C) universal buffer,demonstrating that the universal buffer is more suitable for HIER with the PD-L1 antibody. Nuclear counterstaining was conducted with haematoxylin (blue). Scale bar 20 µm.


A
PDL1
PDL1 Glucagon
PDL1 Glucagon INS
PDL1 Glucagon INS DAPI
B
T1D (ICI) sc41
T1D (IDI) E560
No diabetes 333/66
PDL1
PDL1 Glucagon
PDL1 Glucagon INS
PDL1 Glucagon Insulin

C

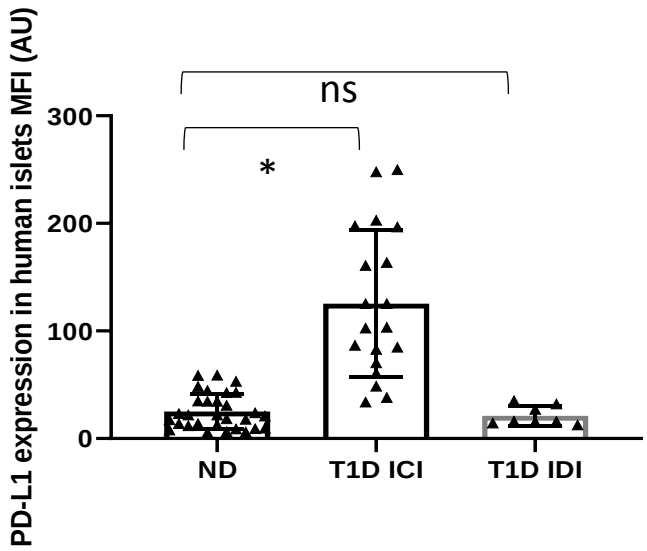


Figure 2-4 PD-L1 expression is elevated in the insulin-containing islets (ICI) of individuals with type 1 diabetes, but not in insulin-deficient islets (IDI) or in islets from individuals without diabetes. (A) Representative immunostaining of PD-L1 within an islet from a donor with acutepancreatitis as a control donor for analysing PD-L1 in an inflammatory condition; PD-L1(green); glucagon (red); insulin (light blue); DAPI (dark blue). Scale bar 20 µm. (B) Immunostaining of PD-L1 is shown in representative islets from individuals without diabetes (lower panels) and those with T1D (middle and upper panels). The upper panels represent an IDI and the middle panels represent an ICI. Scale bar 20 µm. (C) Quantification of PD-L1 expression by MFI analysis in islets from individuals without diabetes (n=25 islets across 5 donor cases), and individuals with diabetes: ICIs (n=19 islets across 4 donor cases) and IDIs (n=8 islets across 2 donor cases) . Analysis was processed using the standard LASX Leica software platform. Statistical analysis was conducted using two-way ANOVA (*$p<0.05$; ns = non-significant).

2.3.2 Interferons regulate PD-L1 expression in β-cells

Our colleagues at the University of Brussels (group of Professor Decio Eizirik) determined that IFN-α, and IFN-γ (but not IL-1β) induced PD-L1 mRNA expression in EndoC-βH1 cells maintained in tissue culture (Colli et al., 2018). In the context of T1D, IFN-α can be released from endocrine cells following enteroviral infection (Chehadeh et al., 2000) and IFN-γ can be released from infiltrating immune cells (Foulis et al., 1991, Morgan et al., 2014). In keeping with this, both PD-L1 mRNA and protein expression were found to be induced by IFN-α in EndoC-βH1 cells and in human islets (Colli et al., 2018). They also determined that PD-L1 expression is dependent on the JAK/STAT1/STAT2 signalling pathway (Figure 2-2) and siRNA knockdown studies confirmed that IFN signalling via IRF1 could regulate the expression of PD-L1.

2.3.3 IRF1 is highly expressed in individuals with T1D and positively correlates insulin expression

Downstream of the JAK/STAT pathway is an important transcription factor, IRF1, which has been implicated in modulating cytokine-induced β-cell death in NOD mice (Gysemans et al., 2009) and whose expression is increased in T1D (Lundberg et al., 2016). However, the protein expression of IRF1 in human pancreas samples has not been investigated. Therefore, the expression of IRF1 was investigated in eight T1D samples (6228-04, DiViD1 to 6 and E560) and five non-diabetic controls (6099-01, 6047-02, 6095-04, 6024-08 and 6160-02). The expression of IRF1 in the endocrine cells was explored as well as any relationship with immune markers, in an effort to identify the inflammatory environment in which IRF1 might be present.

Using IF, a marked increase in IRF1 expression was observed in the nuclei of cells within ICIs in subjects with T1D (Figure 2-5). IRF1 was not identified in any

donors without T1D. In donors with T1D, IRF1 was found to be present in all eight tissue samples and was frequently found in residual ICIs but not IDIs. IRF1 expression was observed in both α- and β- cells, as determined by expression in the nucleus of endocrine cells expressing insulin (blue) and glucagon (red). This occurred across the EADB, nPOD and DiViD organ donor collections. IRF1 was not evenly distributed around islets, but often found concentrated in a particular area. Examination of all donor cases revealed that IDIs did not express IRF1.

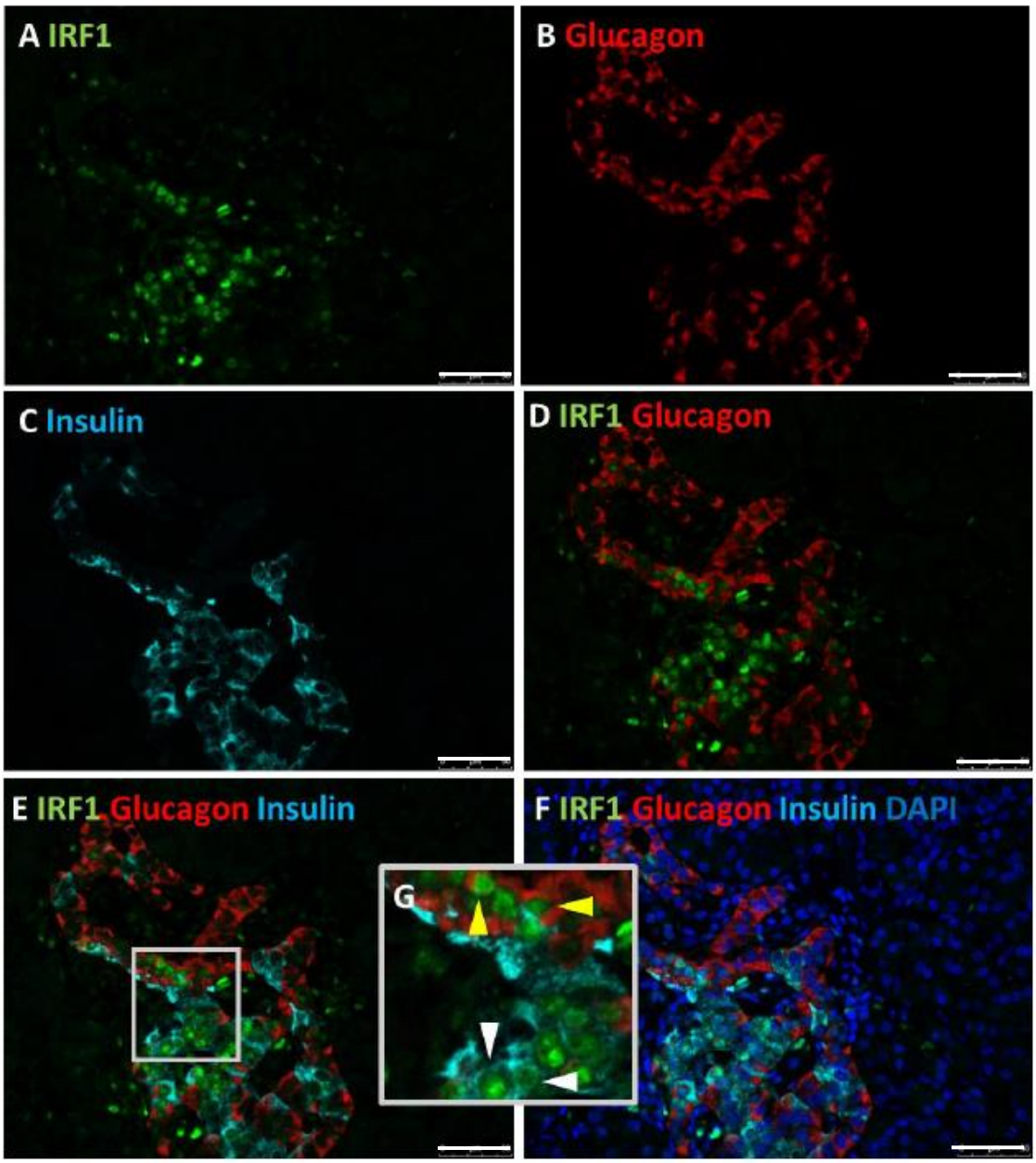


Figure 2-5 Expression of insulin, glucagon and IRF1 in a residual insulin containing islet from a donor with T1D. Representative images of (A) IRF1 (green) (B) glucagon (red) (C) insulin (blue) (D) overlay of IRF1/glucagon (E) overlay of IRF1/glucagon/insulin (F) including nuclei (dark blue) (G) IRF1 positive β-cells (white arrow) and IRF1 positive α-cells (yellow arrows). Scale bar 50 μm. With thanks to Dr. Chaffey.

2.3.4 Expression of IRF1 correlates with immune markers; CD45 and PD-L1

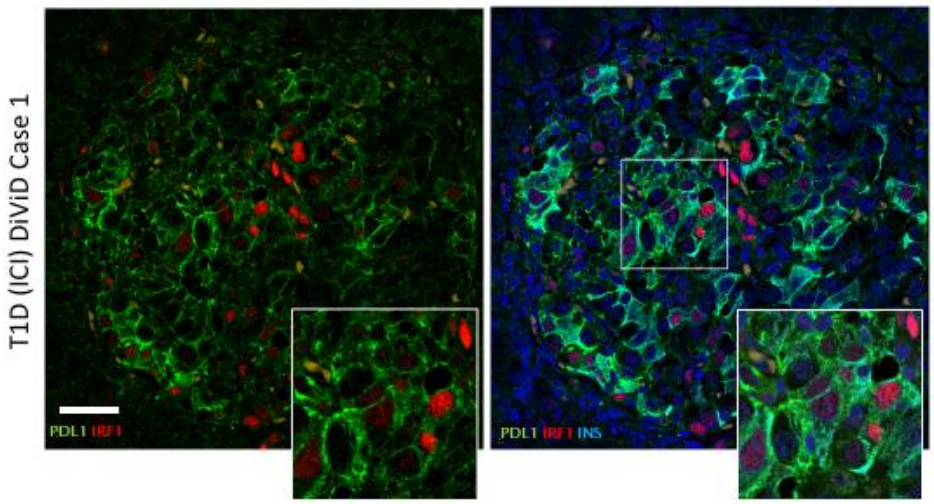
Given that we knew expression of PD-L1 could be regulated by IFN signalling via IRF1, it was investigated whether IRF1 correlated with PD-L1 in T1D. Pancreas section from the DiViD collection (six T1D individuals) were immunostained for PD-L1 and IRF1. In keeping with previous findings, the expression of PD-L1 and IRF1 were both upregulated in islets that contained insulin (Figure 2-6A). The numbers of ICIs with each of the following categories were assessed; PDL1+/IRF1+; PDL1-/IRF1+; PDL1+/IRF-; PDL1-/IRF1- (Figure 2-2B). PD-L1 and IRF1 were frequently observed in the same islets (Figure 2-6A) and interestingly, PD-L1 expression was never observed in the absence of IRF1. There were only a select few number of islets where IRF1 was present but no PD-L1. This indicates that the expression of PD-L1 correlates with IRF1.

As increased PD-L1 levels had previously been associated with IFN-γ, which can also be released from infiltrating T cells in T1D (Foulis et al., 1987) I sought to determine whether there was a relationship between the presence of infiltrating CD8+ T cells and PD-L1/IRF1 expression. Additionally, PD-L1's binding partner PD-1 is known to be expressed on the surface of CD8+ T cells, and therefore, I wanted to determine if increased levels of PD-L1 matched increasing levels of CD8+ T cells.

The data collected above were combined with data regarding the level of immune infiltration (as assessed by CD8 staining) from serially stained sections in each of the DiViD donor samples. Nearby serial FFBE sections had been previously immunostained for CD8, CD20 and insulin. This allowed for calculation of the average CD8+ T cell count per ICI (conducted by Dr. Pia Leete) for each of the donors. The presence or absence of PD-L1 and IRF1 in ICIs was then correlated with the degree of immune infiltration through

assessment by two independent observers (JH and JC). The presence of PD-L1 and IRF1 was positively correlated with level of immune infiltration (Figure 2-6C). Taken together, these data suggest that there is a striking correlation between IRF1 and PD-L1 expression and that this is further associated with the level of immune cell infiltration in each islet.

A



B

DiViD case No.	PDL1+ only ICIs No. islets (%)	IRF1+ only ICIs No. islets (%)	PDL1+IRF1+ ICIs No. islets (%)	PDL1- IRF1- ICIs No. islets (%)	PDL1 & IRF1 concordance No. islets (%)	Average CD8+ T cell/ islet section
1	0 (0%)	3 (20%)	10 (66.7%)	2 (13.3%)	12 (80%)	24.0±3.7
2	0 (0%)	1 (6.7%)	3 (20%)	11 (73.3%)	14 (93.3%)	5.1±0.9
3	0 (0%)	0 (0%)	14 (93.3%)	1 (6.7%)	15 (100%)	21.8±2.9
4	0 (0%)	1 (6.7%)	11 (73.3%)	3 (20%)	14 (93.3%)	9.1±1.4
5	0 (0%)	0 (0%)	12 (80%)	3 (20%)	15 (100%)	12.2±1.8
6	0 (0%)	1 (6.7%)	2 (13.3%)	12 (80%)	14 (93.3%)	4.8±1.0

C

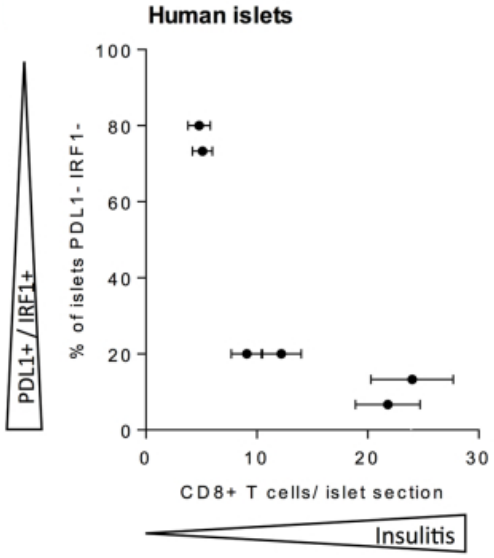


Figure 2-6 IRF1 and PD-L1 expression in the islets of a T1D donor and evidence of a positive correlation with degree of immune infiltrate. (A) Representative images of an ICI from a T1D donor demonstrating PD-L1 (green), IRF1 (red), insulin (light blue) and nuclei (DAPI; dark blue).The insets show the regions highlighted in each islet with a white box at higher magnification. (B) 15 islets were selected at random from each of 6 individuals with T1D (from the DiViD) cohort) and analysed for the proportion that were immunonegative for both PD-L1 and IRF1, or were immunopositive for PD-L1 alone, IRF1 alone or both PD-L1 and IRF1. Average number indicates the mean average (C) The proportion of islets that were immunonegative for PD-L1 and IRF1 was scored in relation to islet inflammation measured as the mean number of CD8+ T-cells/ ICI present in each individual patient studied. Scale bar is 20 µm.

2.3.5 PD-1 is expressed on T cells of young T1D individuals

After successfully demonstrating that PD-L1 is expressed in the islets of individuals with T1D, I sought to investigate if its molecular binding partner PD-1 was present on T-cells either within or around the islets of donors with T1D. All T cells express PD-1 during activation, such that it is a marker of effector T cells. Furthermore, PD-1 is expressed by subsets of tolerant T cells, regulatory T cells, T follicular helper cells, T follicular regulatory cells and memory T cells. PD-1 is also found on several other cell types including B cells, NK cells, some myeloid cells and cancer cells. For the PD-1 pathway, context is key, so issues of timing, location, T cell differentiation state, antigen burden, inflammation levels, metabolic state and other factors influence the functional outcome of PD-1 engagement (Sharpe and Pauken, 2018). Without PD-1, excessive immune-mediated tissue damage can lead to terrible consequences for the host. In addition, PD-1 plays crucial roles in central and peripheral T cell tolerance, assisting in the defence of self-tissues from autoimmune responses.

As with previous experiments, I optimised the PD-1 antibody using immunohistochemistry in human tonsil and pancreatic tissue. As shown in Figure 2-7, PD-1 is expressed in and around the germinal centres of the tonsil but was not present in human islets.

Expression of PD-1 was then examined in donors with T1D, comparing a young (sc115) vs. older (E560) subject and controls without diabetes Tissue sections were immunostained for PD-1, CD8, insulin and cell nuclei. While several immune cells are known to express PD-1 on their cell surface, CD8 was chosen because a previous a study examining pancreatic islets using histology found evidence of increased loss of β-cells, increased loss of insulin staining and

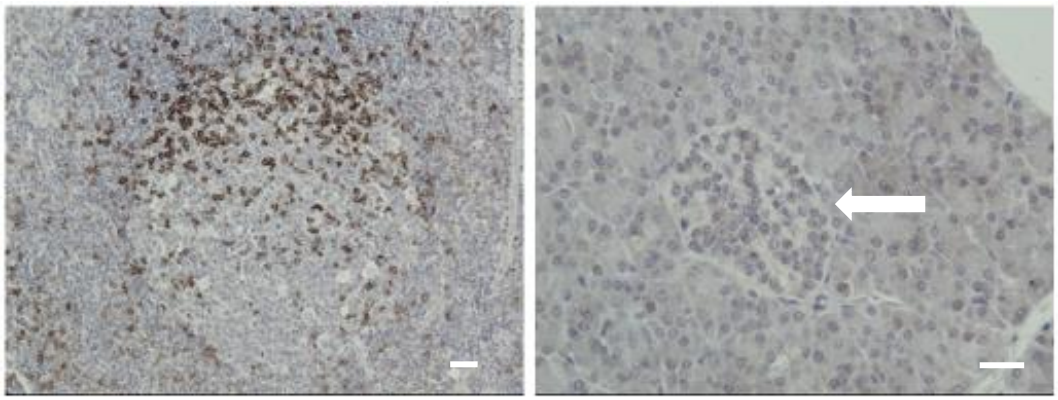


Figure 2-7 PD-1 expression in human tonsil and pancreatic tissue. IHC was conducted with anti-PD-1 antibody, a citrate buffer for HIER, an HRP-linked secondary antibody and DAB staining (brown). Left image illustrates PD-1 expression (brown) in the germinal centres of the tonsil. Right image illustrates the absence of PD-1 expression in the islet (white arrow) of an adult with T1D (E560). Nuclear counterstaining was conducted with haematoxylin (blue). Scale 50 µm.

increased immune infiltration with increased numbers of intra-islet $CD8^+$ T cells and B cells in individuals who developed T1D (Leete et al., 2016). The adult and non-diabetic donors did not appear to express PD-1 in or around the islets, nor was there evidence of PD-1 expression in the exocrine tissue. The younger donor (sc115) had both CD8+ and PD-1+ cells around a small number of ICIs (Figure 2-8AB). There was no expression of PD-1 present in the IDIs of the younger donor.

To confirm the presence of PD-1 in T1D subjects, I chose to use a second PD-1 antibody raised in an alternative animal (mouse) to confirm that the two anti-PD-1 antibodies stained positive for the same immune cells. Initial optimisation of the second PD-1 antibody was conducted in human tonsil tissue and was shown to correlate with the expression of both CD8+ and CD3+ on immune cells (Figure 2-9). It is important to highlight that not all CD8+ or CD3+ cells were PD-1+, and combinations included CD8+/CD3+/PD-1+; CD8+/CD3-/PD-1+; CD8-/CD3+/PD-1+; and CD3+/CD8+/PD-1- cells. The mAb PD-1 antibody was then tested in the older T1D subject (E560), but as seen in the previous experiment, no PD-1 immunostaining was observed.

Both PD-1 antibodies were tested in the young subject (sc115) and found to immunostain the same cells. Since immunostaining was limited to four antibodies, and the insulin antibody verifies islet identity, no immune cell markers were used in this experiment but the co-staining of PD-1 antibodies is likely occurring on immune cells (Figure 2-10A).

In addition to cells outside the islets (most likely immune cells), the PD-1 antibody raised in rabbit (rAb) also stained insulin positive islet cells (Figure 2-10B). To eliminate the possibility of non-specific reactivity with the insulin

antibody (raised in guinea pig), staining was conducted again in sc115 tissue using a different, biotin-tagged insulin antibody (Figure 2-10B). However, the rAb immunostaining was still observed in insulin positive cells, even after fluorescent channels (555 and 647) on the Leica software were manually adjusted to prevent overlap of fluorescence emission from one detector channel into another. This off target staining, was perplexing and did not occur in *the previous experiments using case sc115 or affect the staining in case E560.* To confirm that PD-1 expression was unexpected in human pancreatic beta cells, I reviewed the expression of PD-1 in RNAseq datasets (Hastoy et al., 2018, Blodgett et al., 2015, Russell et al., 2019). The expression of PDCD1, the gene encoding PD-1, was barely detectable. As such I was forced to conclude that this second antibody was prone to non-specific staining of islet endocrine cells.

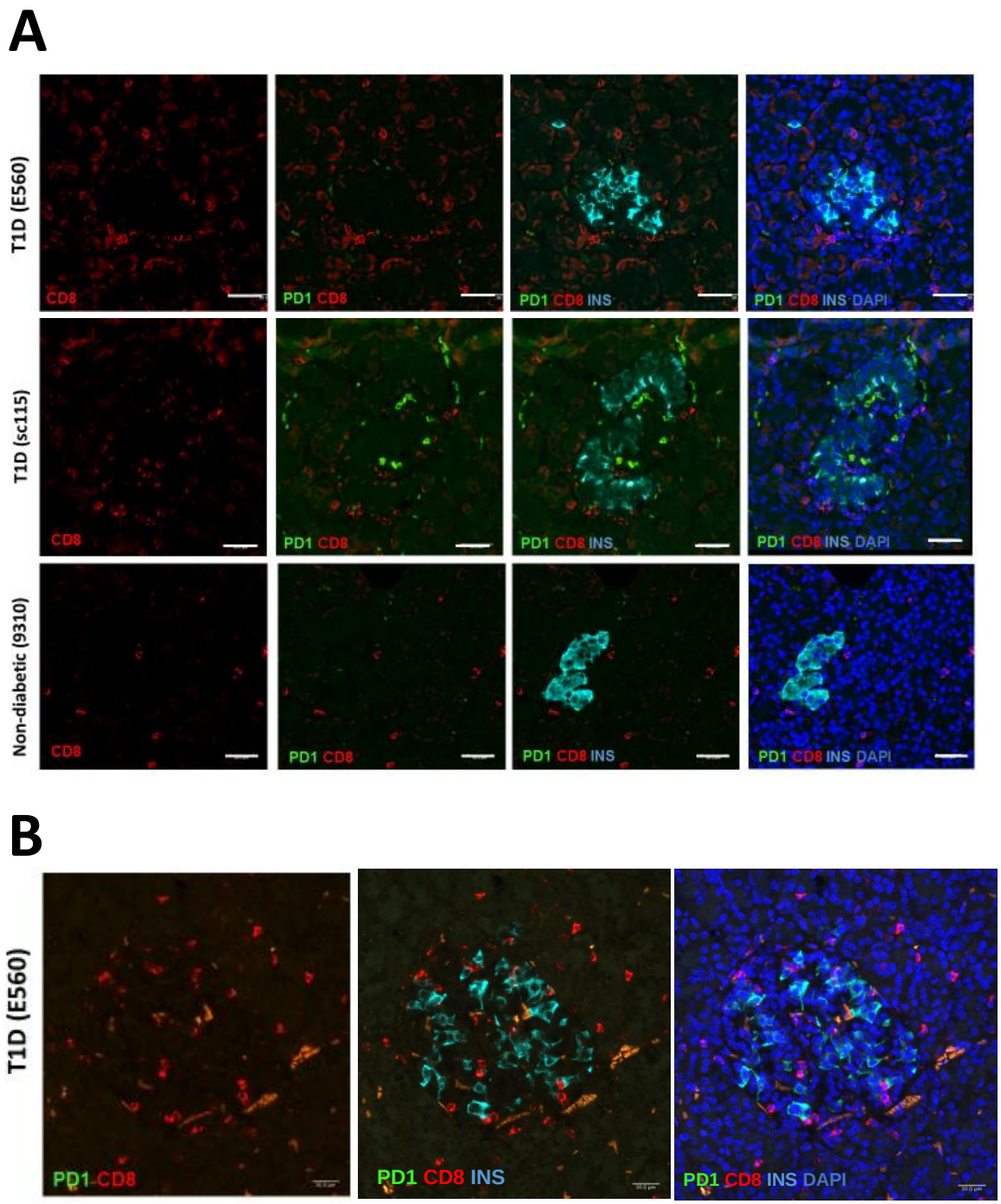


Figure 2-8 PD-1 is expressed in CD8+ T-cells of young T1D subject. (A) Representative images of an islet from an older adult T1D subject (upper panels), a young T1D subject (middle panels), and a non-diabetic subject (lower panels). Immunostaining for CD8 (red), PD-1 (green), insulin (light blue) and nuclei (dapi; dark blue). (B) Second islet representation of PD-1 (green) CD8 (red) and insulin (light blue) in the older adult T1D subject. Orange staining demonstrates presence of red blood cell autofluorescence Scale bar is 30 µm.

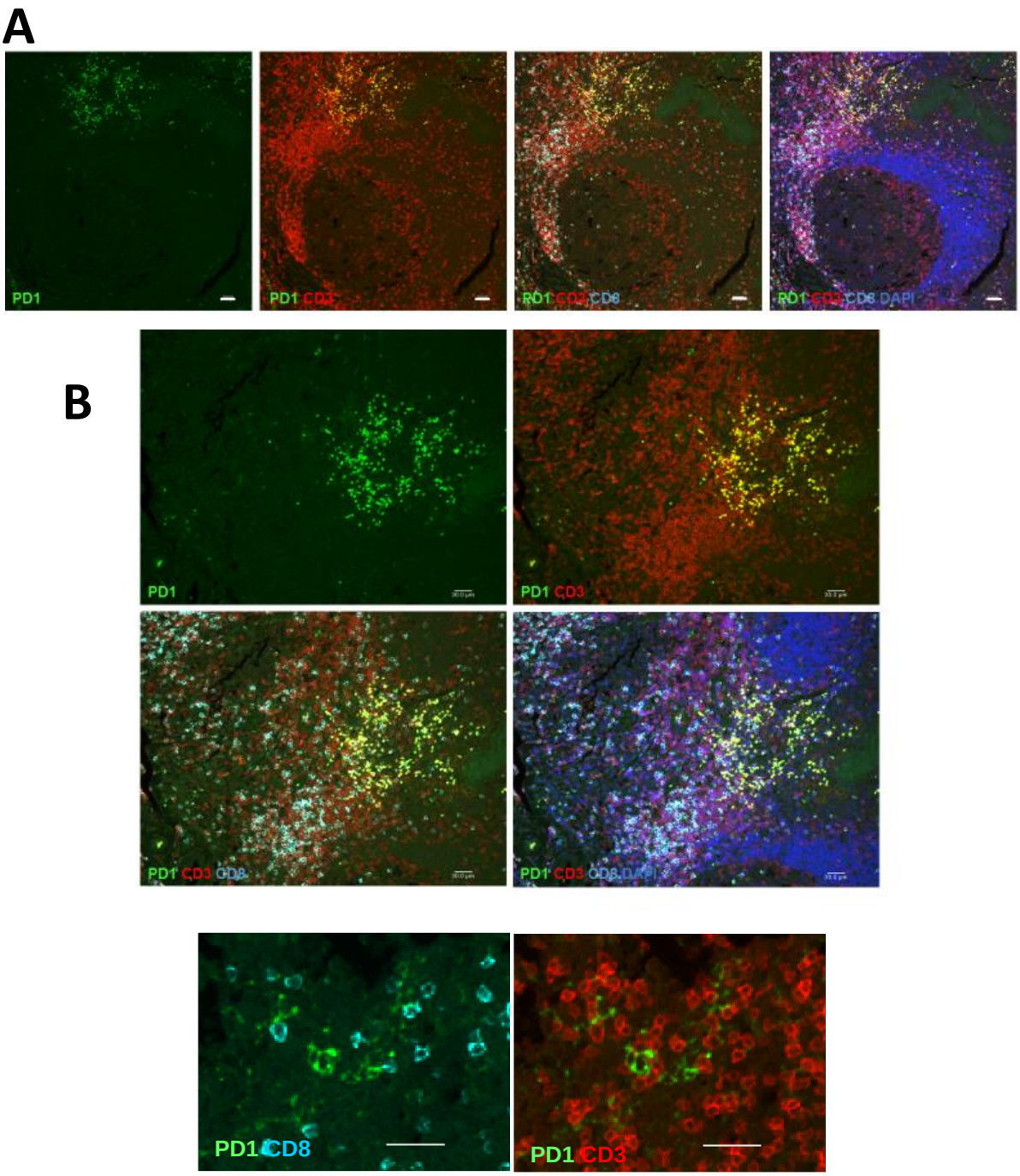


Figure 2-9 PD-1 expression correlates with T cells in human tonsil.

Representative images of human tonsil from one subject using IF. (A) Immunostaining for CD3+ (red), PD-1 (green), CD8+ (light blue) and nuclei (dapi; dark blue) demonstrates co-localisation of PD-1 with subsets of T cells. (B) PD-1 expression was found with either CD3+ or CD8+ T cells, or CD3+/CD8+ T cells, but not all T cells co-localise with PD-1 expression. Scale bar is 30 µm.

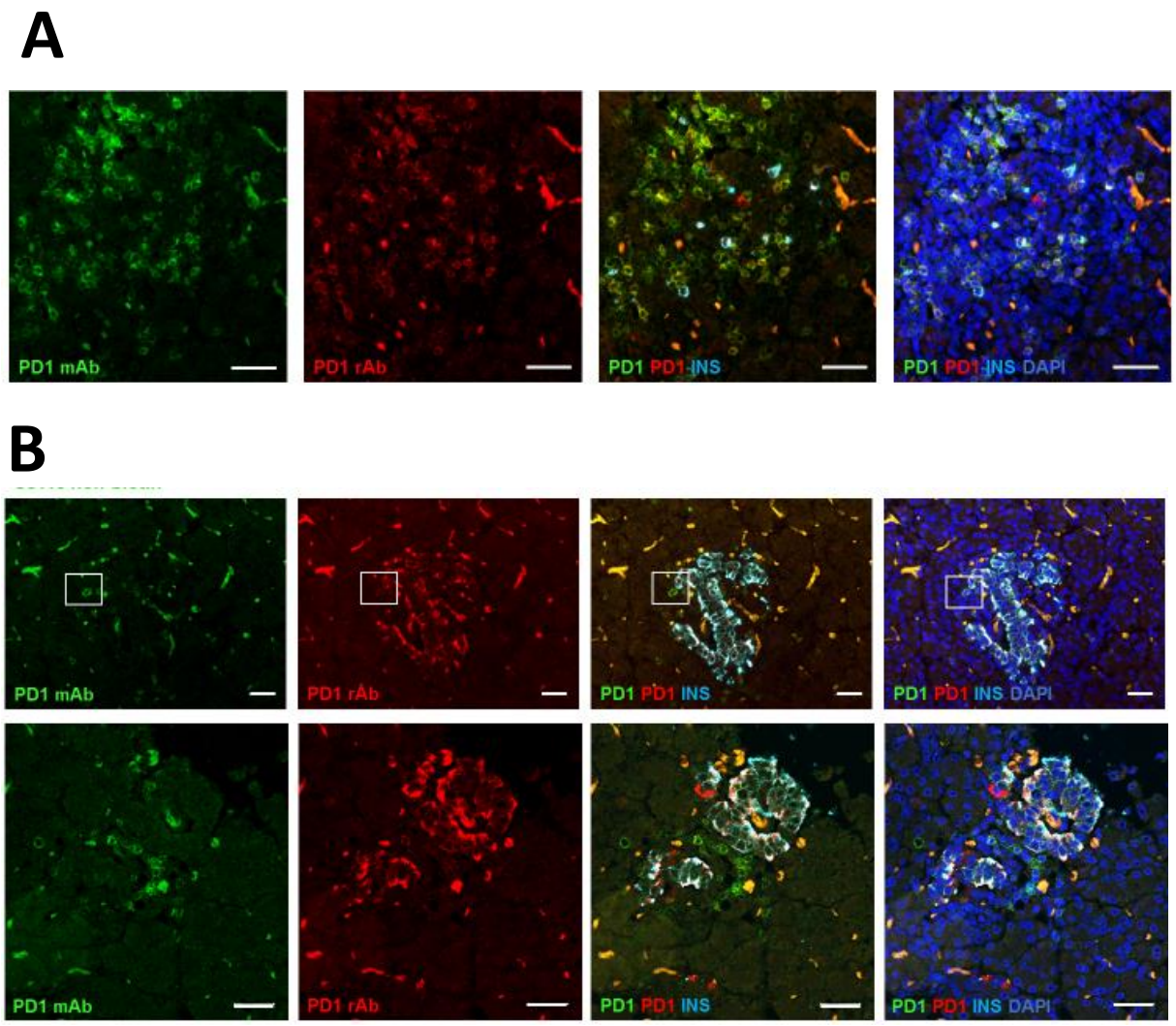


Figure 2-10 PD-1 is expressed in pancreatic tissue of young subject with T1D. Representative images of human islets from one young T1D subject (sc115) using IF (A) Immunostaining for two PD-1 antibodies raised in mouse (mAb; green) and rabbit (rAb; red), insulin (light blue) and nuclei (dark blue). (B) Immunostaining for PD-1 (mAb; green), PD-1 (rAb; red) and non-biotin tagged insulin antibody (top panel), and biotin tagged insulin antibody (lower panel) in the islets of subject sc115. White box in upper panels indicates PD-1+ cell on the periphery of the islet. Scale bar is 30 µm.

2.3.6 HLA-E is expressed in the islets of individuals with T1D

To determine if there were additional proteins present in the islet that could act similarly to PD-1, the presence of HLA-E was investigated. HLA-E was chosen because of its known function as a checkpoint inhibitor but also because collaborators at the University of Brussels observed an upregulation of HLA-E mRNA expression in EndoC-βH1 cells upon treatment with IFN-α (Creelan and Antonia, 2019, Colli et al., 2018).

HLA-E is an immune checkpoint inhibitor and a conserved nonclassical HLA class I molecule. It plays a role in the immune response and is expressed by myeloid dendritic cells, B cells, natural killer (NK) cells, and T cells, and is highly expressed in neutrophils (Grant et al., 2020, Coupel et al., 2007) The conventional role of HLA-E is thought to be its interaction with the innate immune system, and its role in presenting self-peptides derived from the leader sequence of other HLA molecules to NK cells (Tomasec et al., 2000, Braud et al., 1998). However, recent evidence has demonstrated it can also act in a similar fashion to classical HLA-I molecules by presenting pathogen-derived peptides to the adaptive immune system, namely CD8+ T cells (Joosten et al., 2016). HLA-E is regarded as a checkpoint inhibitor because it can negatively affect the cytotoxic function of both CD8+ T cells and NK cells by engaging the inhibitory receptor NKG2A/CD94. As seen with PD-L1, HLA-E is commonly overexpressed on tumour cells, and expression is inducible by inflammatory cytokines (Le Dréan et al., 1998, Rapaport et al., 2015, André et al., 2018, Gooden et al., 2011)

To investigate the presence of HLA-E in pancreatic tissue, I immunostained tissue sections with HLA-E, insulin, and glucagon in subjects with diabetes and age-matched controls. To analyse the expression of HLA-E in ICIs, thirty ICIs

were randomly selected from six independent individuals with T1D (five islets per individual; sc115, sc41, E308, E560, DiViD1 and DiViD3), and thirty ICIs were randomly selected from six individuals without diabetes (five islets per individual; 21/89, 18490, PM191, 146/66, 333/66, and 9310). Tissue sections were sourced from the EADB and DiViD collections. HLA-E was clearly expressed within the islets of individuals with T1D but was absent in the age-matched controls. Interestingly, HLA-E expression was mainly localised to the α-cells as seen by co-staining with glucagon, with occasional expression in the β-cells (Figure 2-11AC). To determine if HLA-E was expressed in the islets absent of insulin in T1D individuals (a sign of β-cell loss and dysfunction), twenty IDIs were randomly selected from four individuals with T1D. In the absence of insulin, HLA-E was not expressed in any endocrine cell, α-cell or β-cell. Mean fluorescence intensity analysis (MFI) demonstrated that expression of HLA-E was significantly higher ($p<0.001$) in ICIs from T1D individuals compared to the age-matched controls, and there was no significant difference between HLA-E expression in the T1D IDIs and age-matched controls (Figure 2-11B).

When analysing the ICIs from T1D individuals, it was noted that a small number of cells within the islets stained positive for HLA-E but were both insulin and glucagon negative. Therefore, to determine if co-localisation was occurring with the δ-cells, immunostaining was conducted with insulin and, glucagon (cyan) and somatostatin (red) in a tissue section from an individual with T1D. After analysing all the ICIs within this individual, it was determined that HLA-E (green) did not colocalise with somatostatin (Figure 2-11D).

After reviewing the EADB donor cases, I found that the T1D individuals with higher expression of HLA-E generally had higher expression levels of PD-L1, and thus immune infiltrate.

Interestingly, in one islet a cell was found to have a nucleus 2x the size of the surrounding nuclei. This islet was depleted in insulin, but the enlarged cell was found to correlate with both insulin and HLA-E expression (Figure 2-12). I found this intriguing because of the breadth of islets observed throughout these studies, I have only seen an enlarged nucleus once.

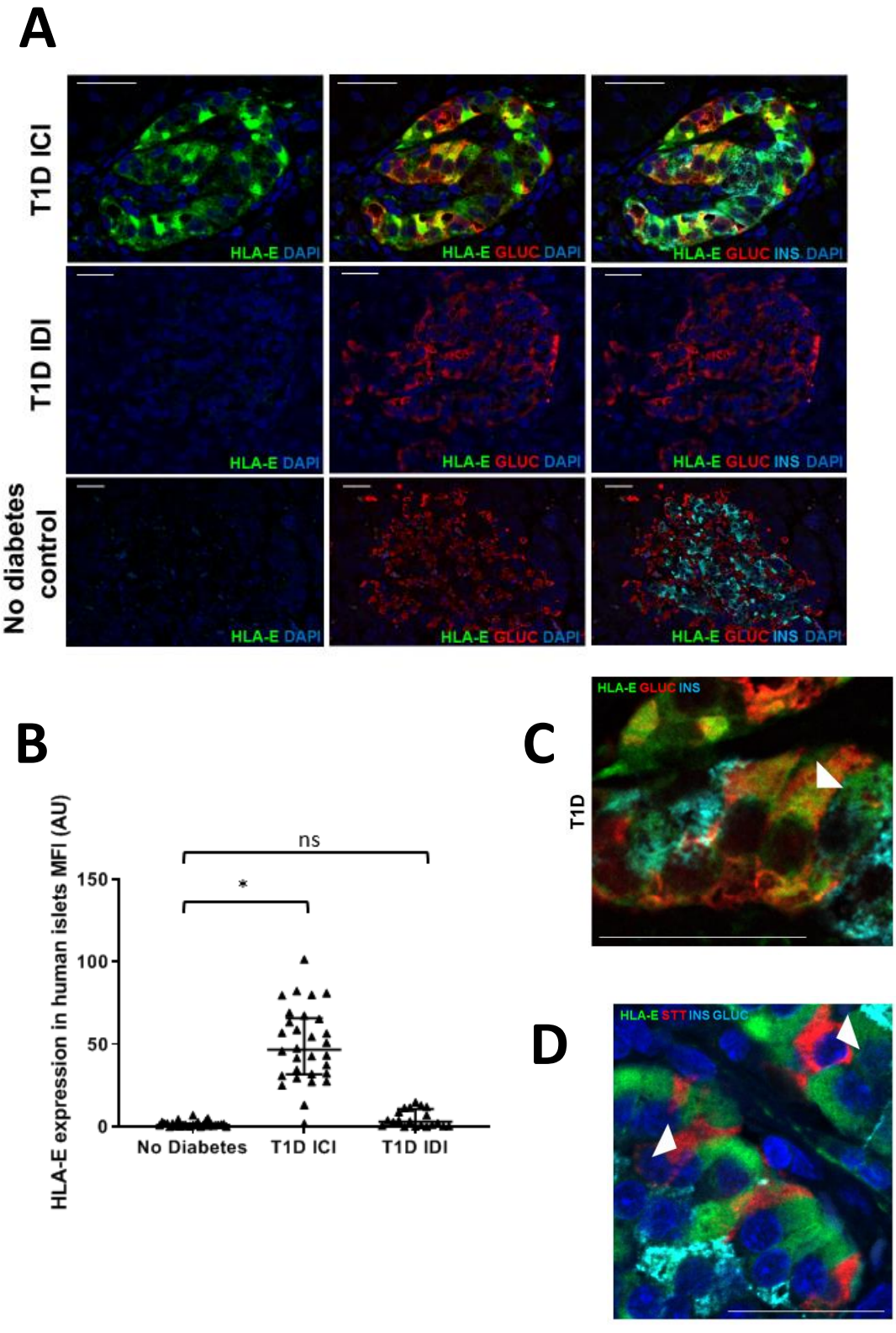

A
T1D ICI
T1D IDI
No diabetes control
HLA-E DAPI
HLA-E GLUC DAPI
HLA-E GLUC INS DAPI
B
HLA-E expression in human islets MFI (AU)
ns
*
150
100
50
0
No Diabetes
T1D ICI
T1D IDI
C
T1D
HLA-E GLUC INS
D
HLA-E STT INS GLUC

Figure 2-11 HLA-E is expressed in pancreatic islets of T1D individuals. Representative images of human islets from adult T1D subjects using IF (A) Immunostaining for HLA-E (green), glucagon (red) insulin (light blue) and nulcei (DAPI; dark blue) in representative islets from individuals with or without diabetes. The top panels represent an insulin-containing islet (ICI) and the middle panels represent an insulin-deficient islet (IDI) from T1D sample DiViD 3. Thelower panels represent an islet from a non-diabeticdonor (EADB sample 333/66). Scale bar 20 μm. (B) The MFI analysis of HLA-E expression. 30 ICIs from 6 separate individuals with T1D (5 islets per individual), 20 IDIs from 4 independent individuals with T1D (5 islets per individual), and 30 ICIs from 6 separate individuals without diabetes (5 islets per individual) were analysed. Values are median ± interquartile range; ANOVA with Bonferroni correction for multiple comparisons, AU (arbitrary units), *p< 0.0001, ns = (non-significant). (C) Higher magnification image demonstrating that HLA-E (green) localizes predominantly to α-cells (glucagon; red) in a T1D donor islet but is also expressed in β-cells (insulin; light blue). (D) Higher magnification image demonstrating that HLA-E (green) is not expressed on the δ -cells (somatostatin; red). Scale bar 30 μm.

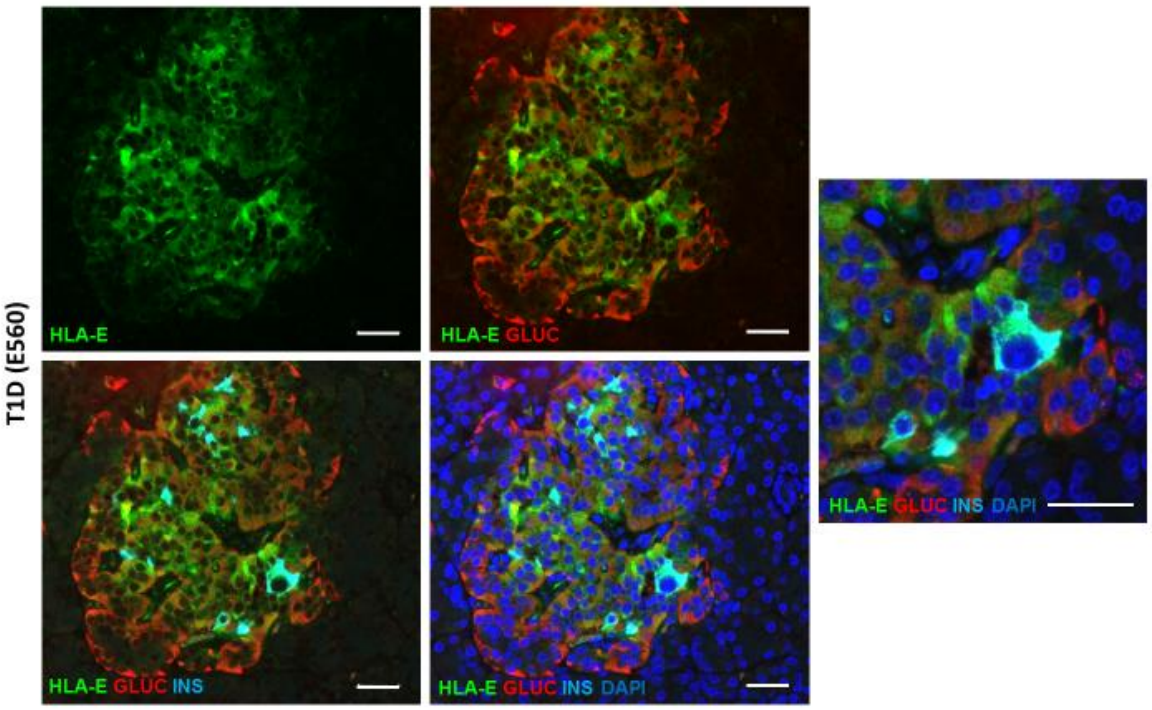


Figure 2-12 HLA-E expression in an insulin containing β-cell with an enlarged nucleus. Representative images of a human islet from an adult T1D subject (E560) using IF. Immunostaining for HLA-E (green), glucagon (red) insulin (light blue), and nuclei (DAPI; dark blue). Image on the right is of higher magnification demonstrating the enlarged nucleus (dark blue) of a cell expressing insulin (light blue) and HLA-E (green). Scale bar 30 μm.

2.3.7 TIGIT is not detected in the pancreas tissue of individuals with T1D

Alongside PD-1, T cell Immunoreceptor with Ig and ITIM domains (TIGIT) has also been identified as an immune checkpoint protein in T cells, and both proteins are often found co-expressed on tumour antigen specific CD8+ T cells and tumour infiltrating lymphocytes (Sharpe and Pauken, 2018). In humans, expression of TIGIT can be found on NK cells and T cells, including CD4+ T cells, CD8+ T cells and T_{regs}(Harjunpää and Guillerey, 2020). TIGIT was identified as a protein of interest for this study because it negatively regulates T cell function by competitively binding CD155, a ligand shared by CD226 (Attanasio and Wherry, 2016, Johnston et al., 2014). Binding between TIGIT and CD155 results in increased IL-10 production and decreased IL-12 production by dendritic cells, leading to suppression of T-cell activation and promotion of T-cell exhaustion (Yu et al., 2009). When co-expressed with PD-1, it can be used as a marker of T-cell exhaustion, particularly in cases of chronic diseases such as HIV (Chew et al., 2016). T cell exhaustion is caused by chronic antigenic stimulation and exposure to chronic inflammation, T cell exhaustion results in a progressive loss of effector functions and potential over time. There are subsets of exhausted T cells that differ in functionality (Sharpe and Pauken, 2018).

T-cell exhaustion plays a pertinent role in the pathology of T1D with a recent study showing that T1D individuals who were designated as slow disease progressors had an increased proportion of autoreactive, islet-specific CD8+ T cells expressing an ‘exhausted’ phenotype. Elsewhere, research groups are currently looking for new strategies that could increase the numbers of exhausted CD8+ T cells to potentially delay the progression of autoimmune diseases (McKinney et al., 2015, Kwong et al., 2021). The role of TIGIT in T1D

pathogenesis remains unclear, and to date there have been no studies analysing its expression in human pancreatic tissue, therefore, using IF I sought to determine if TIGIT was present in individuals with T1D.

Firstly, to optimise methods for antigen retrieval, I conducted IHC on human tonsil tissue and immunostained for TIGIT. TIGIT staining was positive both within and around the germinal centres (Figure 2-12). TIGIT is normally expressed on the surface of cells, which was observed in the tonsil tissue, but there were also areas where staining appeared to localise within the cell.

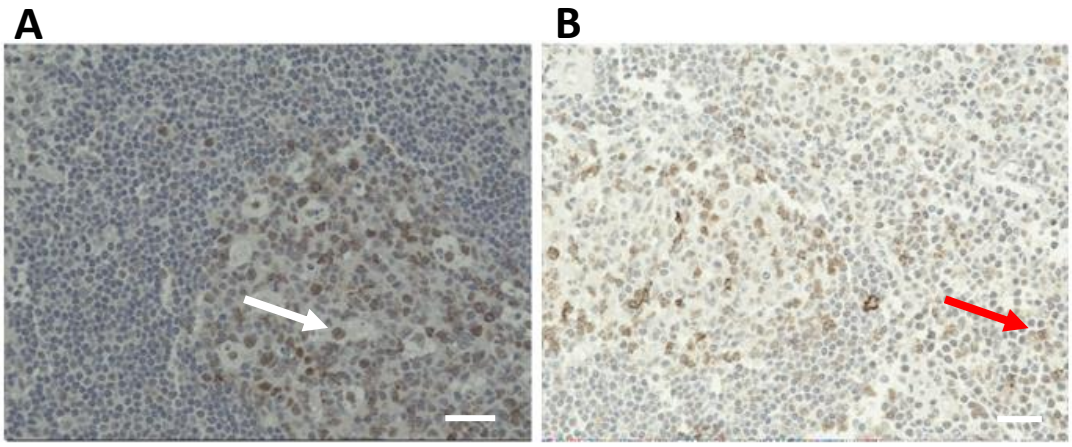


Figure 2-13 TIGIT expression in human tonsil. Two representative images showing expression of TIGIT across a section of tonsil. IHC was conducted with anti-TIGIT antibody, citrate buffer for HIER, HRP-linked secondary antibody and DAB staining (brown) on tonsil tissue sections. Expression of TIGIT (brown) was observed on both the surface of cells and within the cell (towards the middle of the cell). (A) TIGIT is expressed within the germinal centres (GC) of the tonsil (as indicated by the white arrow) (B) as well as at the GC periphery (as indicated by the red arrow). Nuclear counterstaining was conducted with haematoxylin (blue). Scale bar 50 µm.

To determine if TIGIT was expressed in the pancreas of individuals with T1D, tissue sections from an individual with T1D, which previously stained positive for PD-L1 and PD-1 expression, were chosen (sc115). Pancreatic tissue samples from an adult T1D individual and age-matched controls were also tested. Immunostaining was conducted with TIGIT, CD3 (a marker of T cells), and insulin. TIGIT was not observed to be present in the individuals with T1D despite the presence of CD3+ T cells (Figure 2-14).Previous experiments have demonstrated that sc115 contains high levels of insulitis and inflammation but despite this TIGIT was not observed. This may be due to inefficient antibody staining but IF was conducted using high concentrations of antibody and a tyramide signal amplification kit, which is designed to amplify definition of low-abundance targets and offers sensitivity 10-200 times that of standard IHC methods. Therefore, it is likely that TIGIT is not present in this individual, but antibody sensitivity issues cannot be ruled out.

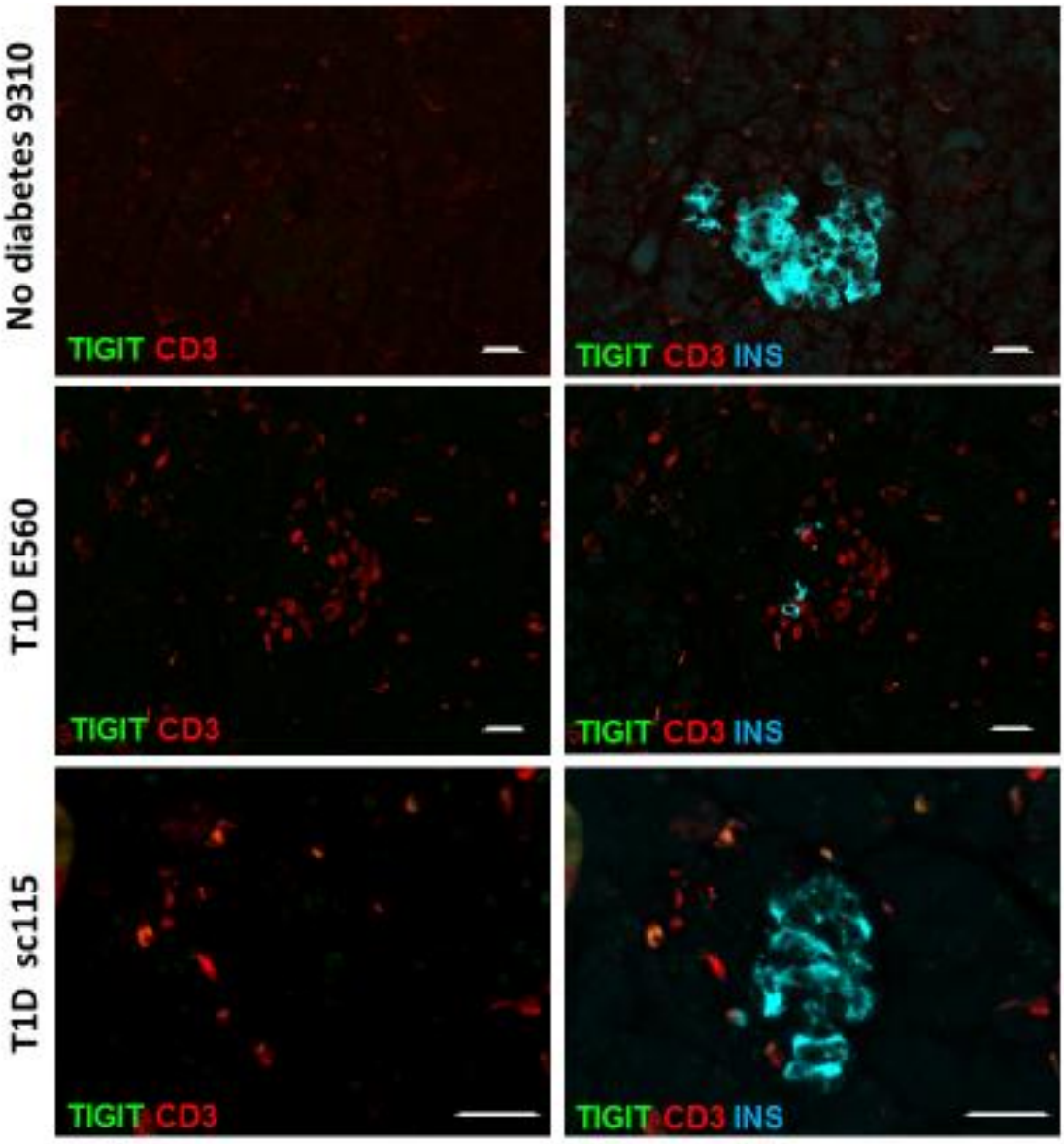


Figure 2-14 TIGIT is not expressed on T cells in subject with T1D.

Representative images of islets using IF from a non-diabetic subject (upper panels; 9310), an older T1D subject with long-duration diabetes (middle panels; E560) and a younger T1D subject with early onset diabetes (lower panels; sc115). Immunostaining for TIGIT (green), CD3+ (red) and insulin (light blue) demonstrates the absence of TIGIT within or around insulin containing islets. Scale bar 30 μm.

2.4 Discussion

A crucial function of the PD-1/PD-L1 pathway is to limit immunopathological responses to host tissues by promoting the resolution of inflammation. Without negative regulatory mechanisms in place, CD8+ T cell responses are not adequately controlled and severe immunopathology can result (Sharpe and Pauken, 2018). This study has shown that PD-L1 is expressed in the islets of individuals with T1D. PD-L1 expression was observed in the insulin producing β-cells of ICIs but was absent in islets deficient of insulin and individuals with no diabetes. Expression was observed in both young and adult T1D individuals, indicating that PD-L1 is present throughout duration of disease and is regulated by presence or loss of insulin producing β-cells. The absence of PD-L1 in IDIs suggests cells that express PD-L1 have been destroyed, or the signalling pathways that regulate PD-L1 have been downregulated. Additionally, following the destruction of the β-cells, the immune cells may downregulate cytokine release and leave the islet area resulting in the removal of factors that induce PD-L1 expression in cells. Importantly, not all islets expressed similar levels of PD-L1 and this may be due to the fact that islets show heterogeneity across the pancreas (Dybala and Hara, 2019), there are also variable levels of insulitis across islets in T1D as well.

An IFN signature is known to be present in the pancreas of individuals with T1D. The expression of PD-L1 positively correlated with IRF1, which was expected since IFNs are known to regulate expression of PD-L1 via IRF1. No islets were observed to express PD-L1 in the absence of IRF1. Additionally, these two proteins were found to correlate with the level of CD8+ immune infiltration of islets in T1D individuals. It has previously been established that the infiltrating CD8+T cells are IFN-γ positive (Foulis et al., 1991), therefore one

could hypothesise that the infiltrating CD8+ T cells releasing IFNs, drives the upregulation of IRF1 and subsequent PD-L1. This suggests that individuals experiencing more aggressive immune phenotypes in T1D (Leete et al., 2020) have β-cells that express higher levels of PD-L1. This would be expected since PD-L1 functions to inhibit T cell functions. This could provide evidence of the β-cells attempting to protect themselves from the immune attack. However, in the younger individuals they have fewer residual β-cells, so this protection, at least under these circumstances is not sufficient to prevent their demise.

Colleagues at the University of Brussels demonstrated that PD-L1 can be induced by type I and II IFNs in human islets and EndoC-βH1 cells , and PD-L1 induced via IFN-α was dependant on the JAK/STAT pathway, particularly STAT1/STAT2/IRF1. PD-L1 mRNA expression was found to be 5-8 times higher in islets from recent-onset T1D individual as compared to islets of an individual who had long-duration T1D and healthy controls (Mastracci et al., 2018, Colli et al., 2020). The discovery of PD-L1 expression on β-cells via induction of JAK/STAT pathways raises potential questions about the use of JAK inhibitors as anti-diabetic agents. Inhibitors of STATs are of interest because of the close association between STAT1 with hyperexpression of HLA Class I in T1D (Richardson et al., 2016), which may promote cytotoxic T cell killing of β-cells. Together these occur uniquely in patients with T1D, thereby contributing to their selective susceptibility to autoimmune-mediated destruction and hyperexpression of classical HLA Class-I. These drugs are under evaluation for the therapy of autoimmune diseases (Fleischmann et al., 2012) and have been suggested as potential therapies for T1D (Coomans de Brachène et al., 2018). Careful evaluation should be considered when pursuing these strategies since it may be that blocking JAK also prevents PD-L1

expression on β-cells. Ideally, drugs should be designed to prevent IFN-α induced HLA class I up-regulation and ER stress without blocking a protective PD-L1 response.

In 1991, Foulis et al. analysed 87 islets from twelve individuals with T1D and found around 40% of the lymphocytes identified contained IFN-γ. Put together with more recent studies (Willcox et al., 2009, Richardson et al., 2014b, Morgan et al., 2014, Leete et al., 2016) these data may explain why not all islets in individuals with T1D expressed PD-L1, as IFN expression is variable. Further studies are needed to clarify the intersection between IFNs contributing to the development of diabetes, as seen in early trials of IFN treatment for Hepatitis C and cancer (Guerci et al., 1994, Oka et al., 2011), and IFN pathways contributing to the delay of the immune assault in autoimmune diseases.

During insulitis, there is likely to be a molecular dialogue between the infiltrating immune cells and the resident β-cells within a landscape of pro and anti-inflammatory cytokine release. As mentioned earlier, the commonly held belief is that T1D is triggered by dysfunction of the immune system, however some argue that β-cells can also provoke an immune attack from a breakdown in normal β-cell processes (Ilonen et al., 2019). Indeed, β-cells have been described as complicit in their own demise by hyperexpressing HLA-I and promoting engagement and targeting by antigen-specific CD8+ cytotoxic T cells (Richardson et al., 2016). This Chapter demonstrates that following the immune attack, a dialogue between the β-cells and immune cells persists and includes signals that can promote β-cell demise (HLA class I) and potentially protect β-cells (expression of PD-L1). If the outcome of PD-L1 expression in islets mirrors what is seen in tumour microenvironments, then PD-L1 expression may act as a defensive mechanism for the islets by slowing the immune assault. This

proposal is supported by previous studies using NOD mice demonstrating that increased PD-L1 expression is associated with long-term survival of β-cells (Rui et al., 2017, Au et al., 2021).

The present data may help to explain why some individuals develop T1D after immunotherapy with anti-PD-1 or anti-PD-L1 inhibitors. It is unclear whether T1D arising after immunotherapy in patients with cancer is caused by pre-existing autoimmune responses that is worsened by checkpoint inhibitors or whether T1D is caused by priming of naïve autoreactive lymphocytes. However, it may be the case that individuals who developed T1D were genetically susceptible to T1D (Thomas et al., 2018) but had no symptoms of the disease. Prior to treatment, the interaction between PD-L1 and PD-1 may have been successful at regulating T cell expression and preventing destruction of β-cells through cytotoxic T cell killing. Traditionally, T1D was thought to be a condition that affected the young, but presentation of symptoms and diagnosis of T1D can occur past the age of 50 (Thomas et al., 2018). This is relevant because most cancer patients are diagnosed later in life.

The clinical relevance of this chapter is that some individuals may benefit from genetic screening prior to immunotherapy to determine their risk for developing T1D and sensitivity to immunotherapy. Additionally, there is great interest in translating PD-1 pathway engagement to induce tolerance in patients with autoimmunity and transplant rejection. There is clinical opportunity in engaging PD-1-mediated inhibitory signals to promote tolerance. A feature of the PD-1 pathway that makes it an attractive therapeutic target is that PD-1 is only expressed on T cells that are responding to antigen, thus, PD-1 pathway modulation could selectively affect certain immune responses as opposed to broadly affecting host immune cells.

Previous studies have proposed that timing has a crucial role in determining whether PD-1 blockade leads to a breakdown in self-tolerance. In NOD mice, PD-1 blockade induced diabetes more rapidly in older mice than in younger mice and this was suggested to be due to increased T cell infiltration and PD-L1 expression seen in the pancreas of older NOD mice (Ansari et al., 2003). This chapter found expression of PD-L1 in both young and older individuals with T1D, which is significant because T1D in younger individuals has been found to display a more aggressive immune phenotype (Leete et al., 2020). This highlights the many challenges with trying to translate findings from murine models into humans. Additional studies are needed to define the stability of dysfunctional T cell states to determine when PD-1/PD-L1 immunotherapy may (or may not) reprogram these cells.

PD-1 expression appeared to be present in the younger but not older T1D individual, but no conclusions can be drawn at this stage as further analysis on additional T1D samples would be required. The young subject had PD-1+/CD8+ cells closely proximal to the insulin producing β-cells. Tissue samples from young individuals within the EADB collection are known to have a different immune profile by comparison with the older individuals, with more extensive insulitis and higher levels of immune destruction of β-cells. The sample that had positive immunostaining for PD-1 also strongly immunostained for PD-L1 and IRF1. Further studies were intended to investigate additional T1D subject tissues but experiments were halted due to the COVID-19 pandemic.

The unexpected observation that the second PD-1 antibody exhibited positive staining within insulin positive cells was perplexing and steps were undertaken to determine if this was due to cross-reactivity or fluorescence bleed-through. These studies did not resolve the issue and therefore, to confirm that PD-1

expression was unexpected in human pancreatic β-cells, I reviewed the expression of PD-1 in RNAseq datasets (Blodgett et al., 2015, Hastoy et al., 2018, Russell et al., 2019). The expression of PDCD1, the gene encoding PD-1, was barely detectable. As such I was forced to conclude that this second antibody was prone to non-specific staining of islet endocrine cells. However, despite staining for insulin, both PD-1 antibodies positively immunostained the same cells within the exocrine and near the β-cells. Therefore, I have confidence that there was true PD-1 staining on T cells within the young T1D case.

Islet expression of non-classical HLA Class I (HLA-E) is a key new finding during development of T1D, and fits into the wider narrative of a pro-inflammatory environment induced by IFN-α. Colli et al. (2020) successfully demonstrated that HLA-E along with classical HLA Class I types are expressed in EndoC-βH1 cells and human islets upon treatment with IFN-α. Islet HLA class I overexpression is a defining feature of T1D development (Richardson et al., 2016), contributing to the recruitment of autoreactive CD8+ T cells that selectively attack beta cells.

The upregulated expression of HLA-E on α-and β-cells could be a further indication that protective mechanisms are employed by the islet cells when faced with an immune assault and supports the assertion that a molecular dialogue exists between β-cells and influent immune cells. Colleagues at the University of Brussels demonstrated that IRF1 is a regulator of the IFN-α-mediated ‘defence’ responses in β-cells, including PD-L1 and HLA-E responses, but further work would need to be done in elucidating the mechanisms regulating induction of HLA-E expression in α-cells. It may be that they mirror what is seen in β-cells, but this would need to be confirmed (Colli et

al., 2020). Nevertheless, HLA-E on α- cells may explain why α-cells are more resistant to the immune assault.

This study found an absence of TIGIT expression the in pancreas, however further studies would need to conclude these findings. The non-biological reasons for this absence could include sensitivity of the antibody, inadequate methods of antigen retrieval, or quality of tissue samples. Elevated levels of TIGIT are often described in oncology and chronic infection as a marker of T cell exhaustion. T cell exhaustion is characterised by the gradual loss of T cell effector functions as a consequence of continuous and prolonged antigen exposure. Exhausted T cells fail to proliferate, they show a defective pro-inflammatory cytokine response, and display highly up-regulated and sustained expression levels of co-inhibitory receptors, such as PD-1, Lag-3, and TIM-3 (Schorer et al., 2020). TIGIT has been shown to play an important role in modulating immune responses in the context of autoimmunity and cancer (Dixon et al., 2018, Schorer et al., 2020) and is a co-inhibitory receptor that limits anti-tumour and other CD8+ dependent chronic immune responses.

Researchers have previously indicated that pathways related to T-cell exhaustion may play a role in restraining T cells in T1D because it takes months (in mice) to years (in humans) after onset of autoimmunity to develop diabetes. Exhausted CD8+ T cells can be rescued by anti-PD-L1 therapy in chronic infection and cancer settings (Kamphorst et al., 2017). With that in mind, immunotherapy may also potentially contribute to the development of T1D if it rescues CD8+ T cells to promote proliferation and full effector function. Recent data show that T cells in the islets of NOD mice display an exhausted phenotype with high PD-1, TIM-3, TIGIT and TOX expression (Abdelsamed et al., 2020). Plus, transcriptomic profiling of T cells from patients with

autoimmunity showed that a T-cell exhaustion signature correlated with a more benign form of autoimmune disease, indicating that mechanisms associated with T-cell exhaustion may be important in controlling autoimmunity (McKinney et al., 2015).

The phenotype of islet specific CD8+ T cells is heterogeneous with more than one distinct phenotype recognised (Ogura et al., 2018). However, one research group has found that subjects with a greater proportion of exhausted islet-specific CD8+ T cells demonstrate slower progression of T1D, though there have been no additional confirmatory studies yet to prove this observation or to relate it to events occurring in the islets (Linsley and Long, 2019). Additionally, there is no clear evidence yet to differentiate between the possibilities that autoimmune inflammation in older subjects occurs as a consequence of chronic antigen stimulation or via episodic autoantigen presentation.

In conclusion, whilst most research has focused on the processes that attract autoreactive T cells towards the islets, such as upregulation of HLA class I molecules and pro-inflammatory cytokine release, I have successfully demonstrated that islets also have defensive mechanisms to negate destruction by T cells. Further clinical studies are required to investigate how PD-L1 expression on β-cells and HLA-E expression on α-cells can delay the progression of diabetes in humans, and if they play a role in periods of disease remission.

Chapter 3

3 Isolating Extracellular Vesicles from Pancreatic Cells

3.1 Introduction

As shown in **Chapter two**, I established that PD-L1 is expressed on the β-cells of individuals with T1D and correlated with expression of IRF1. This supports further work demonstrating that PD-L1 expression is upregulated by IFNs via the JAK/STAT pathway in both EndoC-βH1 cells and human islets. These findings indicate that β-cells may employ a defensive mechanism to attenuate the immune assault and delay the disease progression of T1D.

Human blood contains several forms of soluble or extracellular PD-L1, such as exosomes and microvesicles. Furthermore, PD-L1 is reported to be carried on extracellular vesicles (EVs) released from metastatic melanoma cells, and stimulation with IFNs can subsequently increase expression and release of PD-L1 (Chen et al., 2018). In a study aiming to identify the presence and role of extracellular PD-L1, exosomes released from tumour cells were found to carry more PD-L1 than other EV subgroups and could suppress the function of CD8 T cells, thus allowing tumour growth and evasion of the immune assault (Chen et al., 2018). Interestingly, the levels of circulating PD-L1 on EVs were also discovered to act as indicators of disease state and could be used as biomarkers to stratify clinical responders from non-responders to anti-PD-1 therapy (Chen et al., 2018). Other studies focusing on head and neck cancer found PD-L1 on EVs correlated with the disease stage and activity (Ludwig et al., 2017). Moreover, experiments using mice lacking either PD-L1 expression or exosome production, or both, proved in principle that PD-L1 on exosomes can systemically suppress immune function *in vivo* (Poggio et al., 2019).

EVs have been researched for their potential use as biomarkers for various disease states (Barile and Vassalli, 2017). Since I had previously shown that

PD-L1 expression in patients with T1D correlated with levels of insulin, IRF1 and immune activity, I sought to determine if PD-L1 could be expressed on EVs released from pancreatic cells. If PD-L1 was largely expressed on insulin producing β-cells, then EVs released from β-cells may carry PD-L1 as a biomarker that correlates with immune activity. Additionally, if PD-L1 is present on EVs released from β-cells, then this may attenuate the immune assault by suppressing the function of infiltrating CD8+ T cells.

Therefore, this chapter aimed to successfully isolate EVs from pancreatic cells and determine if PD-L1 expression was present in extracellular form. To achieve this, EVs would be isolated from 1.1B4, Endo-CβH1 and PANC-1 cell lines and treated with IFNs to determine if the signalling pathways regulating PD-L1, as outlined in **Chapter two**, were also regulating extracellular forms of PD-L1 expression.

3.1.2 Isolating Extracellular Vesicles following Tissue Culture

As outlined in the introduction, various types of cells release vesicles of different sizes into the extracellular environment. The processes determining rate of exit from the cell and proportion of vesicle subpopulations released are not well understood but most likely vary depending on the cell of origin. Isolating EVs from cell and animal tissue can be incredibly lengthy and difficult (Doyle and Wang, 2019). Researchers and commercial industries have sought to develop high-throughput methods that minimize the co-isolation of protein aggregates, however there is often a trade-off between length of protocol and EV population purity.

As an example, some commercial products use chemicals such as PEG to isolate EVs. Thermo Fisher's™ Total Exosome Isolation Reagent operates by using water-excluding polymers to settle exosomes out of biological fluids for

collection at low-speed centrifugation. This expensive product is more suited for processing low volume cultures (e.g. 10-15 mL of serum). However, if a researcher was isolating EVs from large volumes of cell culture supernatant (>30 ml) more efficient and economic methods are required.

Several commercial options have been developed that apply different methods of isolation. These include gel-filtration, affinity purification using magnetic beads, and charge neutralisation-based precipitation. Patel et al. (2019) recently compared the efficacy of various EV isolation methods and concluded each method led to different size distributions of EVs. Some of the various methods used are summarised in Table 3-1. Most methods resulted in the isolation of EVs within the accepted size range of exosomes (30-150 nm in size) but others resulted in highly heterogeneous populations containing small and large EVs (>300 nm in size). As a comparator, it was reported that the ‘gold standard’ ultracentrifuge (UC) or differential centrifugation method produced more homogenous populations (size range 120 nm +- 2.65 nm), but homogeneity came at the expense of a more lengthy procedure (Lobb et al., 2015).

Methods	Classical (Ultracentrifugation)	Invitrogen (Precipitation)	101 Bio (PureExo)	MagCapture (Affinity Based)	iZON (Size exclusion)
Principle	Based on density and size under centrifugal force	Differential solubility-based precipitation	By precipitation and targeted filtration for removal of protein contamination	Affinity methods using magnetic beads and phosphatidylserine (PS)-binding protein TIM4	Based on size exclusion chromatography
Pros	Good for exosomes treatment-based study, mass spectrometry analysis, RNA-seq study	Quick, easy and needs small sample size, high yield, good for small volume, RNA-seq study	Quick, easy, yield pure exosomes good for mass spectroscopy RNA-seq study	Single step, easy, no hoarse chemical, good for mass spectroscopy RNA-seq study	Fast, easy, signal kit can be used for all type of sample, little volume is required
Cons	Time consuming, aggregated proteins and nucleic acid may pellet, not good for small volumes	Precipitates non -EV material, not good for mass spectrometry	Good for small volume only	Low yield, good for small volume only	Low concentrated prep, additional method for enrichment required
Time required	3-6 hrs	12-16 hrs	1.5 – 2.0 hrs	4 – 5 hrs	1.5 – 2.0 hrs

Table 3-1. Summary of different EV isolation methods taken from Patel et al. 2019.

To evaluate exosome stability and integrity, Patel et al. (2019) examined the zeta potential of the EV populations collected from the various methods. A higher magnitude of zeta potential indicates higher repulsion between the particles in suspension, suggesting high dispersion stability (Deregibus et al., 2016). UC along with several other commercial models were reported to produce EV samples with good stability, which can also be an indication for good structural integrity. To assess the purity of EV samples, they conducted Western blots and found that each isolation method led to a different signal intensity signal for CD9, a marker of exosomes. They did not observe major differences in RNA quantity and quality, but Mass Spectrometry analysis revealed that protein quality differed between isolation methods. Most commercial reagents were found to be unsuitable for good quality proteomic analysis because the various chemical contaminants (e.g. SDS, high NaCl, polyethylene glycols, fetal bovine serum) hindered the mass spectroscopic study of the proteins. Finally, an assessment of biological quality was conducted by treating pre-cultured MiaPaCa cells with isolated exosomes. After 72 hrs, cell proliferation was measured and all exosomes, except those isolated by the Invitrogen method, had a positive effect on cell proliferation. This suggested that the Invitrogen reagent likely contained cytotoxic chemicals that inhibit cell growth (Patel et al., 2019).

It was also noted that methods with increased numbers of ‘washes’ often led to loss of EV quantity, while precipitation-based methods could lead to precipitation of aggregated proteins or large EVs, which poses potential issues for exosome studies.

In summary, this analysis and similar studies (Doyle and Wang, 2019, Livshits et al., 2015, Brennan et al., 2020) have proven that different methods for EV isolation will

produce variable results in terms of population heterogeneity and purity. There is no blanket protocol for EV isolation and methods must be developed and optimised for the biological fluid from which EVs are extracted and the intended downstream analysis. This indicated that for my studies, I would need to design and optimise my own protocols for isolating EVs from pancreatic cells.

3.1.3 Isolating EVs using Differential Ultracentrifugation

Ultracentrifugation (UC) is a commonly used technique for isolation of microparticles such as viruses, proteins, cells, subcellular components (e.g. mitochondria) and nucleic acids. The principle behind this technique involves the spinning of biological mixtures at ultra-speed causing particles that are heavier than the solvent to sediment. Mixtures are often cell or animal (e.g. serum) culture that contain components of varying sizes and densities, each with different sedimentation rates. Larger particles sediment faster than smaller particles, and thus are removed in the first steps of ultracentrifugation conducted at lower speeds (10,000 x g). Sedimentation of smaller particles requires further centrifugation steps, at higher and longer speeds (100,000 x g).

This technique works well for isolating EVs released from cellular components. The first rounds of centrifugation at 10,000 x g for 30 min separate the apoptotic bodies and cell debris from the EVs. This is followed by two rounds of ultracentrifugation at 100,000 x g for 70 min to isolate the EVs. When there are large differences between sedimentation velocities of particles being separated, then the differential centrifugation can effectively be optimised to obtain high yield and high purity. However, it is less successful when sedimentation rates are smaller in difference between particle fractions. The aim when isolating EVs, is to collect a population that is free from cellular debris, organelles, apoptotic bodies, protein and protein aggregates. The aim of isolating

exosomes, however, is to separate them from larger shedding vesicles of similar sizes (Livshits et al., 2015).

UC methods described in EV studies are often similar, but UC methods can produce differing results depending on the rotor used by the researcher. Special consideration needs to be made to the model of machine and rotor because they affect the sedimentation rates of the vesicles. The theory of ultracentrifugation relies on the geometrical parameters of the rotor, the speed, the duration of centrifugation and the viscosity of the solution. Researchers have published UC protocols without recording the rotor type used and this has subsequently led to significant differences in yield and purity of isolated vesicles when methods are replicated across laboratories (Cvjetkovic et al., 2014).

The physics behind the sedimentation rates are as follows:

There are three balancing forces at play behind the velocity of particles moving through a fluid in a centrifugation tube: centrifugal force, Archimedes floatation force and Stokes viscous drag force.

$$v = g_{eff}\frac{d^2}{18\eta}(\rho - \rho_{solv}) \qquad g_{eff} = \omega^2 R$$

- *geff is the centrifugal force (acceleration)*
- *R is the radius of rotation*
- *ω is the angular frequency of rotation*
- *d is the diameter of the particle*
- *ρ is the mass density of the particle*
- *ρsolv is the density of the medium*
- *η is the viscosity of the medium*

When the rotation speed and medium composition is fixed, then density and morphology determine the velocity of the particle.

Any centrifugation protocol needs to consider; a) the rotor type and b) particle characteristics to be separated. For my research, I conducted all experiments using a swinging bucket rotor Figure 3-1A, as opposed to the fixed-angle rotor Figure 3-1B.

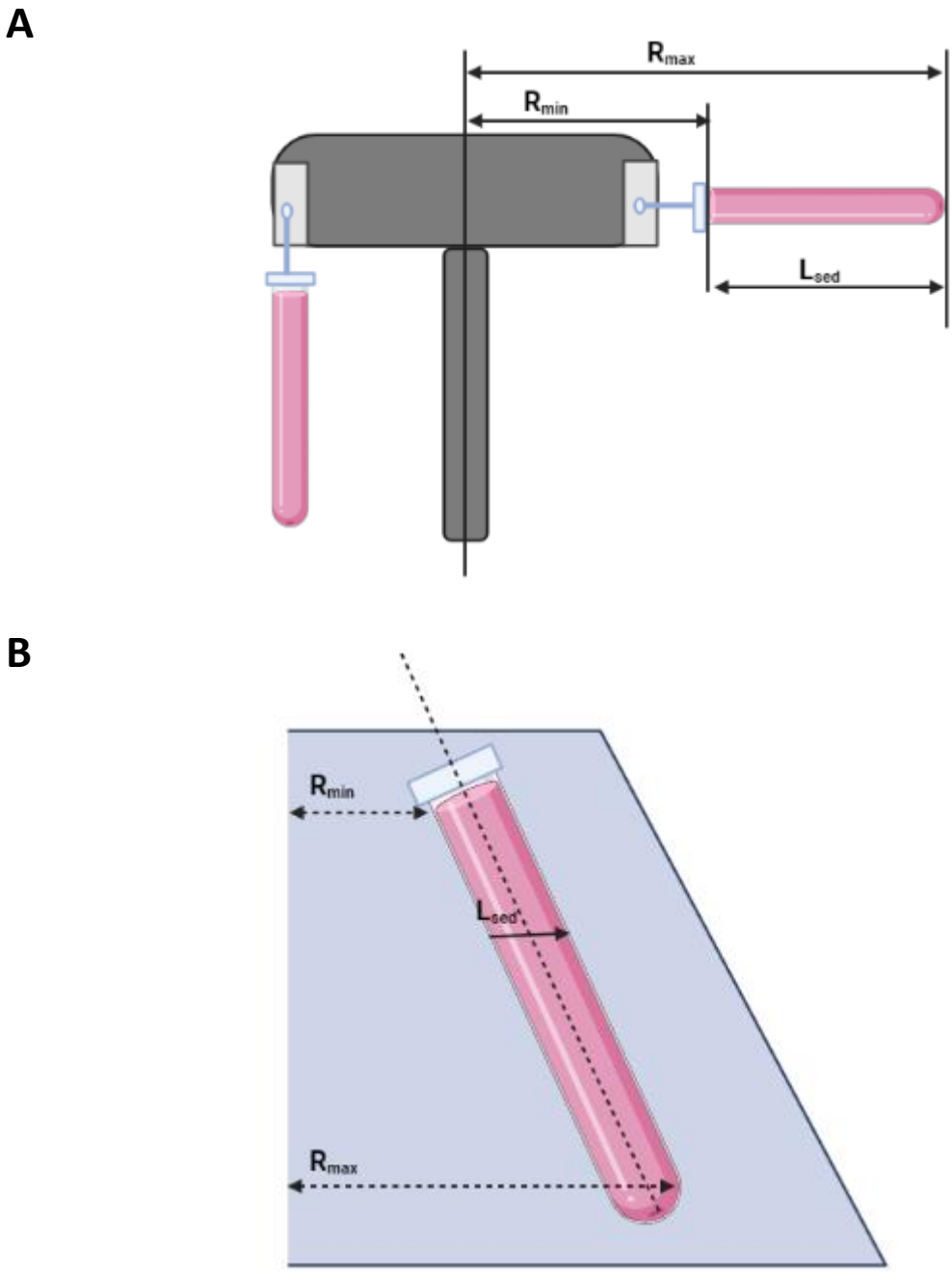


Figure 3-1. Diagram of the two types of rotors used for ultracentrifugation.

(A) The swinging-bucket rotor (B) Fixed angle rotor.

The geometry of the design of swinging bucket rotors means they have different sedimentation properties to fixed-bucket rotors. The Lsed as described in Figure 2-1 is called the sedimentation path length of the rotor and as shown, the Lsed in fixed-angle rotors is shorter. Vesicles become sedimented when they reach the tube bottom for swinging-buckets rotors, or the tube wall opposite the rotation axis for fixed angle rotors. In the fixed-angle rotor, the particles can glide down the slide of the tube to pellet at the bottom, but this cannot be predicted or calculated. This unpredictability means that the pellet may not be compact, which could mean significant loss of sample during isolation.

Livshits et al. 2015 proposed a method to equalize the sedimentation efficiency of UC with different rotors using what they call the “cut-off size” based method. The UC duration needed for complete sedimentation of vesicles of a particular size can be calculated for the specific rotor with a selected rotation speed. They designed an interactive web-calculator available at the following domain: http://vesicles.niifhm.ru/. I used this tool for my own studies to predict what type of EV subpopulations I could isolate using our Thermo Scientific™ TH-641 rotor at velocities of 10,000 x g and 100,000 x g.

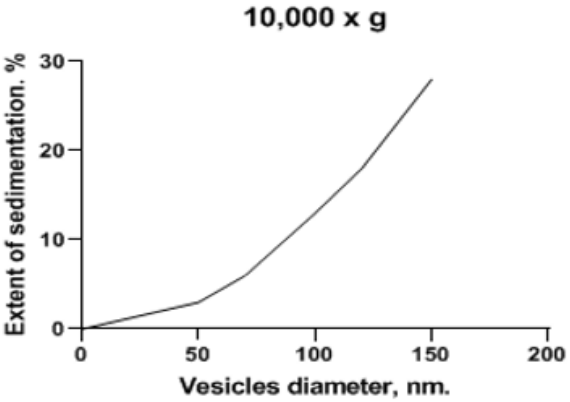
To achieve a complete sedimentation “cut-off” size of 339 nm, a rotation speed of 10,000 x g would need a duration of 30 min (Figure 3-2A).

To achieve a complete sedimentation “cut-off” size of 62 nm, a rotation speed of 100,000 x g would need a duration of 70 min (Figure 3-2B).

In summary, these predictions revealed that centrifuging tissue culture supernatant at 100,000 x g was more likely to produce subpopulations of exosomes and centrifuging tissue culture supernatant at 10,000 x g was more likely to produce subpopulations of larger EVs (>150 nm). This is significant for

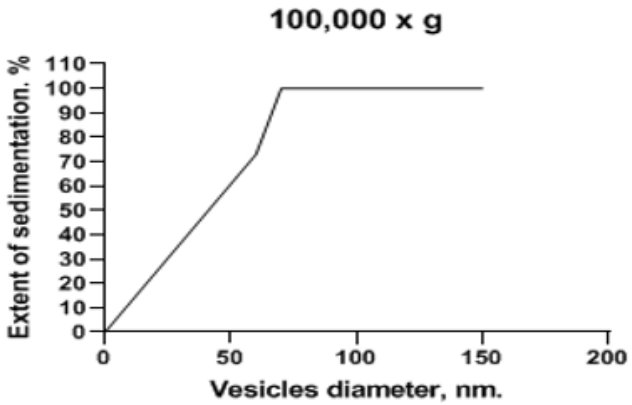
my study because PD-L1 was recorded to be present on both exosomes and larger vesicles (Chen et al. 2018)

A



Vesicle diameter (nm)	Portion of pelleted vesicles
60	4 %
70	8 %
100	15 %
120	22 %
150	33 %

B



Vesicle diameter (nm)	Portion of pelleted vesicles
60	73 %
70	100 %
100	100 %
120	100 %
150	100 %

Figure 3-2 Theoretical prediction of vesicle size and portion of pelleted vesicles after ultracentrifugation using the TH-641 rotor and the "cut-off size" based method. (A) Expected proportions (as % of whole) of pelleted vesicles that are 60 nm, 70 nm, 100 nm, 120 nm, and 150 nm in size from a 30 min UC step at 10,000 x g (B) Expected proportions (as % of whole) of pelleted vesicles that are 60 nm, 70 nm, 100 nm, 120 nm, and 150 nm in size from a 70 min UC step at 100,000 x g. Predictions calculated using the web tool: http://vesicles.niifhm.ru/ developed by Livshits et al. (2015).

3.2 Methods

3.2.1 Cell lines

The cell lines used in these studies were clonal cells of pancreatic lineage:

1.1B4, PANC-1 EndoC-βH1.

1.1B4 cells were reported to be generated by the electrofusion of primary human pancreatic β-cells with the immortal human epithelial PANC-1 cells. The original publication (McClusky et al, The Journal of Biological Chemistry, Vol 286, pp.21982-21992) noted the resulting cells retained the immortal phenotype of the PANC-1 cell lines but were responsive to glucose and secreted insulin (ECACC catalogue number 87092802) (McCluckey et al. 2011). Prior to the creation of this cell line, researchers had no reliable human pancreatic immortalised cell lines that could respond to glucose and produce insulin.

PANC-1 cells are an epithelioid carcinoma cell line derived from the pancreatic duct of a 56 year-old Caucasian male. PANC-1 cells are not glucose-responsive and have an epithelial morphology.

EndoC-βH1cells (provided by Dr. R. Scharfmann, University of Paris, France) (Ravassard et al., 2011) are glucose responsive insulin-secreting cells that reportedly mimic physiological characteristics of the human beta cell. They were created by transforming human foetal pancreatic buds with a SV40LT lentiviral vector containing an insulin promoter. These buds were then transplanted into SCID mice for maturation and the resultant clonal cell line was isolated (Ravassard et al., 2011).

HeLa (CCL-2, ATCC), HEK293 (CRL-1573, ATCC) and neuronal SHSY5Y cell lines (kindly gifted from Prof. Craig Beall's team) were also used as positive controls.

3.2.2 Cell culture

1.1B4 cells were cultured in RPMI 1640 media containing 11.1 mmol/L D-glucose (Gibco), supplemented with 100 U/ml Penicillin and 100 ug/ml Streptomycin (Gibco), 2mmol/L L-glutamine (Sigma) and 10% exosome-free foetal bovine serum (FBS) (Gibco).

PANC-1 cells were cultured in 4.4 mmol/l D-glucose DMEM media (Gibco) supplemented with 100 U/ml Penicillin and 100 ug/ml Streptomycin (Gibco), 2 mmol/l L-glutamine (Sigma) and 10% exosome-free FBS.

1.1B4 and PANC-1 cells were passaged every 3 to 5 days, or when they reached ~80% confluency. All cell lines used are adherent to the culture flask, so passaging involved the dissociation of cells from the flask using Trypsin-EDTA (0.05%) (Gibco). After detachment of the cells, Trypsin-EDTA was neutralised with fresh media (containing FBS) to avoid cell damage. The detached cells were then pelleted by centrifugation at 200 x g for 5 min, the old medium was removed and the pellet resuspended in 10mL fresh medium. Appropriate volumes of suspension was transferred to new culture flasks depending on the desired seeding number, typically 1mL of suspension was combined with 20mL of fresh medium for continued growth of cell population.

EndoC-βH1 cells were cultured in 5.5 mmol/l D-glucose DMEM media (Gibco), supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco), 2% BSA (fraction V, fatty acid free) (SLS), 50 µmol/l β-mercaptoethanol (Sigma), 5.5 µg/l Transferrin (Sigma), 6.7 ng/ml sodium selenite (Sigma), 10 mmol/l nicotinamide (VWR). Cell culture flasks were pre-coated with ‘coating medium’ (4.5 g/l glucose, 1% penicillin / streptomycin, 2 µg/ml fibronectin, 1% ECM) for at least 1 hour prior to seeding freshly split cells. To passage the cells, they

were washed with PBS, then incubated with trypsin / EDTA solution for 3 – 5 min. Once cells were detached from the flask, the trypsin / EDTA solution was neutralised using warmed neutralisation media (80 % PBS, 20 % FBS), and the solution was transferred to a 50 ml centrifuge tube. The cells were pelleted by centrifuging at 700 x g for 5 min. Cells were resuspended in fresh culture medium and seeded at a density of 1.75 x 10^6 cells per ml. EndoC-βH1 cells were sub-cultured every 7-9 days, with 50% of the media refreshed every 3-4 days.

All cell lines were incubated at 37 °C, with 5 % CO2 and 100 % humidity. Before initial seeding, cell lines were mycoplasma negative (MycoAlert™ Mycoplasma Detection Kit (Lonza)).

3.2.3 Cell treatments

Where indicated, cells were pre-treated with human IFNα (pbl assay science, 11200-1) [1000 U/ml], human IFN-γ (R & D Systems) [100 ng/mL], exosome inhibitor GW4869 (Sigma, D1692) [20 μM or 40 μM], and N-SMase Spiroepoxide Inhibitor (Santa Cruz, SC-202721) [5 uM or 10 uM] (Catalano and O'Driscoll, 2019).

3.2.4 Cryo-preservation

Cells were frozen in liquid nitrogen to create stocks of low passage number. Higher passages can often lead to chromosomal and physiological changes within immortalised cell lines. For my studies, cells were not passaged beyond passage number 35.

T25 flasks containing cells at low passage number (ex. passage 4-10) were allowed to reach ~80 % confluency, then the cells were detached from the flask (as explained above) and resuspended in freezing solution (Table 3-4). The cell suspension was aliquoted into cryovials and transported to a -80°C freezer

within CoolCells (BioCision), before being transferred to liquid nitrogen for long term storage.

3.2.5 Whole cell protein extraction

Cells were either detached from flasks and pelleted or directly rinsed in ice cold PBS before lysis buffer (Table 3-4) was applied. To protect the integrity and phosphorylation status of the proteins within the sample, 10 µl/ml of protease inhibitor cocktail (Sigma P1860) and 10 µl/ml of phosphatase inhibitor (cocktails 2 and 3)(Sigma P5726, P0044) were added to the lysis buffer before use. These inhibitors neutralise endogenous proteases and phosphatases respectively.

The sample was incubated for 15 min on ice with the lysis buffer mixture before cells were transferred to a 1.5 ml microcentrifuge tube (Eppendorf). Samples were vortexed briefly 4-5 times before being pelleted by centrifugation at 2,500 x g for 10 min at 4°C. This centrifugation pellets the larger cellular debris such as membranous material, while the proteins are left in the supernatant solution. The supernatant was transferred to a new tube and stored at either -20°C or -80°C.

3.2.6 EV isolation using Thermo Fisher Total Exosome Isolation Kit

EVs from 1.1B4 cells were isolated using the Total Exosome Isolation Kit. Published Thermo Fisher protocols were followed but also briefly summarised here. After incubating a T75 flask of 1.1B4 cells for 72 hrs, the supernatant was removed and placed on ice. Protease inhibitor cocktail (Sigma P1860) and phosphatase inhibitors (cocktails 2 and 3) (Sigma P5726, P0044) were added to the medium which was then centrifuged at 2000 x g for 30 min to remove cellular debris and retained. Exosome isolation reagent was added at a 2:1 ratio, medium to reagent. The solution was then mixed by vortexing and

pipetting up and down until a homogenous solution was formed, the samples were then incubated at 2-8°C overnight. To enrich for EVs, the samples were centrifuged at 10,000 x g for 60 min at 2-8°C. The supernatant was aspirated and discarded, leaving the exosomes in the pellet (not visible in most cases). The pellet was resuspended in lysis buffer over ice for protein extraction and Western blotting, or resuspended in Assay buffer for downstream analysis using Flow Cytometry.

3.2.7 EV isolation using ultracentrifugation

Cells were cultured for 48 hrs before supernatant was removed and placed on ice. Mixtures of protease and phosphatase inhibitors were added to the media as described in section 3.2.5. The media was centrifuged at 2000 x g for 30 min to remove cellular debris and then transferred to ultracentrifuge tubes and loaded onto the TH-641 swinging-bucket rotor (Thermo Scientific™ cat. no. 54296). The swinging-bucket rotor was mounted onto the platform of the ultracentrifuge (Thermo Scientific™ Sorvall™ WX Ultracentrifuge) set to a pre-cooled temperature of 4°C. As calculated in section 3.1.3, to collect larger EV samples (i.e. >150nm), the supernatant was centrifuged at 10,000 x g for 30 min then washed with PBS and centrifuged again . To isolate EV populations consisting of mainly smaller EVs (i.e. <150nm), the supernatant was centrifuged at 100,000 x g for 70 min then washed with PBS and centrifuged again (Greening et al., 2015). The pellet containing the EVs was either resuspended in PBS or lysis buffer depending on downstream analysis.

3.2.8 Extracellular vesicle protein extraction

Once isolated, EVs were resuspended in lysis buffers containing either triton-x-100 or RIPA (Table 3-4). Samples were placed on ice for 15 min before briefly being vortexed.

3.2.9 Protein estimation

Estimation of protein concentrations within samples was done using using a Pherastar plate reader (Pherastar FS, BMG Labtech) and the bicinchoninic acid (BCA) assay (ThermoFisher) (Ernst and Zor, 2010). The BCA assay uses colorimetric analysis of the reduction of copper ions to detect proteins. Firstly, a reaction occurs, called the biuret reaction when copper chelates with protein in an alkaline environment to form a blue colour. A second reaction occurs when the BCA reacts with the reduced copper ion that was formed in the first reaction, leading to a purple colour. The intensity of the purple colour if proportional to the amount of protein in the sample. The reaction can be read in a Pherastar reader at an absorbance measurement of 562 nm.

To normalise the system and create a standard curve against which the samples could be compared, bovine serum albumin (BSA) was used at 0, 200, 400, 600, 800, 1000 and 1200 ug/ml. Using a 96 well plate, each sample was diluted 1:5 and 1:10 and 10 µl of the diluted sample or standard was transferred to a new well in duplicate. BCA reagent was added to these diluted samples and incubated at room temperature on a plate shaker for 10-15 min. During this incubation, the above reactions occur, and the colour of the samples and standards change to a purple. The absorbance was then measured at 562 nm on the Pherastar plate reader, and the protein concentrations of each sample were calculated based on the comparison measurements with the BSA standard curve.

Proteins from isolated EVs were not measured as the overall sample volumes (20 µl) were too small to divide for the BCA assay. Due to the size of EVs and low protein content, detection of EVs using Western blotting required highly

sensitive detection kits, thus whole volumes of EV samples were used for Western blotting.

3.2.10 Gel electrophoresis for whole cell protein preparations

Sodium Dodecyl Sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their electrophoretic mobility and precedes Western blotting or protein staining. Conformation, size and charge of the molecule characterise the electrophoretic mobility of a protein. Proteins of larger sizes can be more easily separated by altering the concentrations of acrylamide in the gels (ex. 10%, 12% etc.). Gels are prepared with specific or gradient concentration percentages depending on the proteins of interest.

Extracted protein samples are combined with LDS buffer (Fisher) (25%) and β-mercaptoethanol (10%). To encourage more even migration down the gel, β-mercaptoethanol is applied to the sample to ‘denature’ the proteins to their primary structures. This reduces the number of disulphide bonds and unfolds proteins to aid the separation of proteins by the factor of size. Sample mixtures are then heated to 70°C for 10 min.

Commercially prepared gels (Bis-Tris 4-12%) were purchased from Invitrogen and placed into an Invitrogen XCell SureLock tank. The tank was then filled with commercially prepared SDS running buffer (Invitrogen) before samples were loaded into the wells of the gels. Wells can typically hold 20 µl of samples before overspill occurs. A commercial protein ladder (Abcam) was also loaded into the first well of the gel to indicate the molecular weights of the migrating proteins. The XCell SureLock system was connected to a voltage pack and commenced a running time of 120 min at 120 V.

3.2.11 Gel electrophoresis for EV samples

Non-reducing conditions are used for EV samples to optimise the conditions for downstream protein detection of tetraspanins such as CD9, CD63, and CD81. In the case of tetraspanins, intact disulphide bonds are important for antibody recognition of conformational epitopes. Extracted samples were combined with LDS buffer (25%), but the denaturing steps of adding β-mercaptoethanol and applying heat were not performed. Samples were loaded into gels as described above.

3.2.12 Transferring protein and polyvinylidene difluoride (PVDF) membrane

Commercially purchased PVDF membrane (Sigma) was soaked in methanol and washed in transfer buffer (Table 3-4) to permeabilise it in preparation for the transfer of proteins from the gel. Sponges and blotting paper are also pre-soaked in transfer buffer before being stacked in the following order: 2 sponges, 1 blotting paper, membrane, gel, 1 blotting paper, 2 sponges. This stack is then placed within a transfer cassette and loaded into the Invitrogen XCell SureLock tank. The tank was then filled two-thirds with water, while the cassette was filled with transfer buffer. The XCell SureLock system was then connected to a voltage pack and commenced running at 30 V for 120 min.

3.2.13 Western Blotting

Western blotting is a widely used technique to analyse protein expression within prepared samples (Burnette, 1981). For these studies, I used Western blotting to analyse protein expression in cell lines, as well as EVs released from cell lines. Following the separation of proteins on SDS-gel and transfer onto a microporous membrane, the proteins can be identified using protein targeted antibodies (Kurien and Scofield, 2015).

Once the transfer of proteins onto the membrane is complete, the membrane was treated with 3-5% milk or 3-5% BSA in TBS-T for 1 hr at room temperature. Primary antibodies (Table 3-2) were prepared in 3-5% milk or 3-5% BSA with TBS-T, and incubated with the membrane for either 1 hr at room temperature or overnight at 4°C.The membrane was washed 3 times in TBS-T for 10-15 min per wash, before secondary antibody (Table 3-3) (in 3-5% milk or 3-5% BSA with TBS-T) was incubated with the membrane for 1 hr at room temperature. To detect the secondary antibody signal, commercial antibodies are conjugated with fluorescent tags or alkaline phosphatase (AP). The membrane was then washed 3 times with TBS-T as before.

3.2.14 Detection of proteins on the membrane

For detection of proteins probed with secondary antibodies conjugated to fluorescent tags, membranes were analysed using the Licor Odyssey CLx scanner. The 700 nm and 800 nm channels were used to detect the conjugated antibodies.

For detection of proteins probed with secondary antibodies conjugated to alkaline phosphatase (AP) tags, detection reagent CDP Star (C0712, Sigma) was applied to the membrane and left to react with the AP tags for 5 min. The membrane was analysed using the Licor C-DiGit scanner under the high sensitivity setting.

Software analysis was performed using the Image Studio Lite Ver 5.2 software.

3.2.15 Stripping the membrane

Stripping membranes is an effective tool for re-probing and detecting additional proteins by removing any bound primary and secondary antibodies. This is particularly useful when probing with antibodies that were raised in the same

species as those used for the initial probing, or when proteins under investigation are of similar molecular weights.

Membranes were washed with TBS-T before being treated with ReBlot Plus Strong Antibody Stripping Solution (10x; #2504, Merck) for 13 min. Membranes were then washed with TBS-T before incubated with a blocking solution (Table 3-4).

3.2.16 Human CD9 Flow Detection CD9 positive isolation protocol

Following isolation of EVs from UC, magnetic beads coated with antibodies specific for EV surface proteins were applied to the sample to enhance purity of EVs.

EVs were pre-enriched using either Total Exosome Isolation reagent (cat. No. 4478359) or ultracentrifugation. Human CD9 Flow Detection (from cell culture) Dynabeads™ were washed in Assay Buffer (Table 3-4) and placed in a magnetic separator for 1-2 min. The buffer was then removed, leaving the CD9 Dynabeads™ in the Eppendorf tube. To mix isolation beads with pre-enriched EV samples, fresh Assay Buffer is transferred into the tube, followed by the pre-enriched EV samples. The EV samples and CD9 Dynabeads™ were incubated at 2–8°C overnight with end-over-end mixing (tilting and rotation). Samples are then centrifuged briefly, before fresh Assay Buffer was added to the tube and placed on the magnetic separator for 1-2 min to wash the bead-bound EVs. The supernatant was then removed from the tube, leaving behind the bead bound EVs. The bead-bound EVs were then resuspended in fresh Assay Buffer.

3.2.17 Flow cytometry analysis after CD9 positive exosome isolation

Typically, flow cytometry uses lasers to measure size and granularity of cells in solution, as well as measure labelled fluorescent signals (McKinnon, 2018). Analysis produced by Flow cytometry is presented as dot plots or histograms,

and each cell is plotted as forward scatter (FSC) vs side scatter (SSC). EVs are too small to be detected by flow cytometry so magnetic beads are used as a solid support for detection by the instrument. Including magnetic beads coated with antibodies specific for exosome surface proteins (CD9) further enhances the purity of the exosome preparation.

After pre-enriched EVs were isolated using magnetic beads, anti-human CD9-RPE clone ML-13 (BD Cat. No. 555372) was added to the bead-bound EV sample. An isotype control was also prepared by adding mouse IgG1-RPE (BD Cat. No. 559320) to a bead-bound EV sample. To stain the samples, the tubes were incubated (away from light) at room temperature for 45 min on an orbital shaker at 1000 rpm. Samples were washed twice by adding Assay Buffer to each tube and then placed on a magnetic separator for 1-2 min before removing the supernatant. Fresh Assay Buffer was added to the tubes before flow cytometry analysis.

Bead-bound EVs were analysed by flow cytometry (BD Accuri C6 Flow Cytometer) using a minimum count of 25,000 events (beads) per sample. The analysis was conducted with as few beads as possible for a strong signal because many exosomes will dock onto each bead and emit a signal when processed through the flow cell.

3.2.18 Nanosight Nanoparticle Tracking Analysis (NTA)

There is a variety of techniques available to quantify EVs but I was trained to use the commonly used Nanosight Nanoparticle Tracking Analysis (NTA) (Malvern Instruments). NTA is highly regarded because it does not rely on detection of a specific marker and the EVs can be suspended in a wide range of solutions (Koritzinsky et al., 2017). NTA utilises the properties of both light scattering and Brownian motion in order to obtain particle size and distribution

of samples in liquid suspension (Dragovic et al., 2011). The NanoSight machine can detect particles with sizes from as little as 10-40 nM, to a maximum size of 1000-2000 nM. The minimum size limit is related to material type, wavelength and power of illumination source, and sensitivity of the camera. The maximum size limit is related to limited Brownian motion. As particles in suspension are pushed across a metalized surface, they are illuminated by a specially configured laser beam. The particles are seen as point scatters moving under Brownian motion. The speed of the particles correlates directly with the particle size. Net flow is detected by the instrument allowing for the addition of a syringe pump to improve measurement quality. The syringe pump slowly pushes the particles through the chamber at a set rate allowing for visualisation of more particles and improving quality and reproducibility of data.

To prime the NanoSight 3000, 0.2 µM filtered PBS (i.e. the diluent) was processed through the machine. It is essential that commercially purchased prefiltered PBS be filtered a second time using a 0.2 mM filter. This is due to the instruments incredible sensitivity as it will measure any contaminating nanoparticles.

Reactive against	Catalogue number	Species reactivity	Dilution	Diluent	Incubation time
HSP90 B1 [GRP94]	HPA008424	Hamster, Human, Mouse	1/500	3% milk	o/n
HSP90 AC88	386040	Mouse, Rat, Sheep, Rabbit, Chicken, Guinea pig, Hamster, Dog, Human, Pig, Fish, Monkey, Chinese hamster	1/500	5% BSA	o/n
CD63 [TS63]	ab59479	Human	1/250	5% milk	o/n
CD9	10626D	Human	1/1000	5% BSA	o/n
CD81	MA1-81488	Goat, Human, Non-human primate, Sheep	1/1000	5% BSA	o/n
ALIX	HPA011905	Human	1/500	5% BSA	o/n
PD-L1 [ABM4E54]	Ab210931	Human	1/500	3% BSA	o/n
PD-L1 [B7-H1, MIH1] PE	12-5983-42	Human	5 uL/Test	Assay Buffer	1 hr
Mouse IgG1-rPE isotype control	559320	Mouse	20 ul/Test	Assay Buffer	1 hr

Table 3-2 Primary antibodies used in Chapter 3.

Reactive against	Catalogue number	Dilution	Diluent	Incubation time
Anti-rabbit AP	3687	1/25,000	5% BSA	1 hr
Anti-mouse AP	A3562	1/25,000	5% BSA	1 hr
DyLight Anti-mouse 680	Abcam 35519	1/5000	5% BSA	1 hr
DyLight Anti-rabbit 800	Abcam SA5-10036	1/5000	5% BSA	1 hr

Table 3-3 Secondary antibodies used in Chapter 3.

Buffers	Recipe
Freezing solution for cells	30ml FBS (Sigma) + 5ml DMSO (Sigma)
Whole cell lysis buffer	20mM Tris, 150mM NaCl, 1mM EDTA, 0.5% or 1% Triton-X (10µl/ml Protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails 2 and 3 (Sigma P5726, P0044))
RIPA Buffer	10mM Tris-HCl, pH 8.0, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140mM NaCl, Dilute with dH2O
BCA	49 parts A : 1 part B Reagent A: bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1M NaOH (pH11.25) Reagent B: 4% (w/v) copper(II) sulphate pentahydrate
Running Buffer	50mM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH7.7
Transfer Buffer	14.4g Glycine, 3g Tris, 0.8L H2O and 0.2L Methanol
LDS buffer	Purchased from Fisher Lithium dodecyl sulphate (pH8.4), Glycerol
Flow cytometry Assay Buffer	PBS with 0.1% BSA, 0.2 uM filtered

Table 3-4 Buffers used in Chapter 3.

3.3 Results

3.3.1 Isolating exosomes from 1.1B4 cells using commercial reagents

As discussed earlier in the chapter, there are many commercial products available that enable methods to enrich or isolate extracellular vesicles and exosomes from plasma or cell culture media. To test whether I could collect EVs released from 1.1B4 cells, I used the Thermo Fisher™ Total Exosome Isolation Kit. The isolation reagent operates by using water-excluding polymers to settle exosomes out of biological fluids for collection at low-speed centrifugation.

1.1B4 cells were chosen as the model of choice because they were reported to resemble β-cells by producing low levels of insulin (McCluskey et al., 2011), and were previously used by our laboratory to study the mechanisms involved in development of persistent Coxsackie viral infections (Chaffey et al. 2021). As previously described in the methods section, 1.1B4 cells were cultured in medium for 48 hours and EVs were isolated using exosome isolation reagent. To determine if the reagent had appropriately isolated EVs of the correct size (ex. exosomes are between 50 – 130 nm) the vesicles were analysed using Nanosight Tracking Analysis (NTA) (Figure 3-3). It was vital to take special care in diluting vesicle samples in commercial filtered PBS before analysis. Unfiltered diluents, particularly H_2O, contain significant levels of contaminants that can skew the analysis (Figure 3-3C).

A representative graph from NTA is demonstrated in Figure 3-4. Five individual ‘frames’ or runs are captured on the software. Each frame is 60 sec long and records the sample flow (containing the EVs) as it passes underneath the laser. The results are presented as vesicle size (diameter of the vesicle) versus concentration (particles/ml). The mean value is then calculated and portrayed

as the black line on the graph. The red lines indicate areas with high variance from the mean. The vesicle sizes at the peak points indicate what subpopulations of EVs are present, for example exosome populations would have a peak point between 50-130 nm.

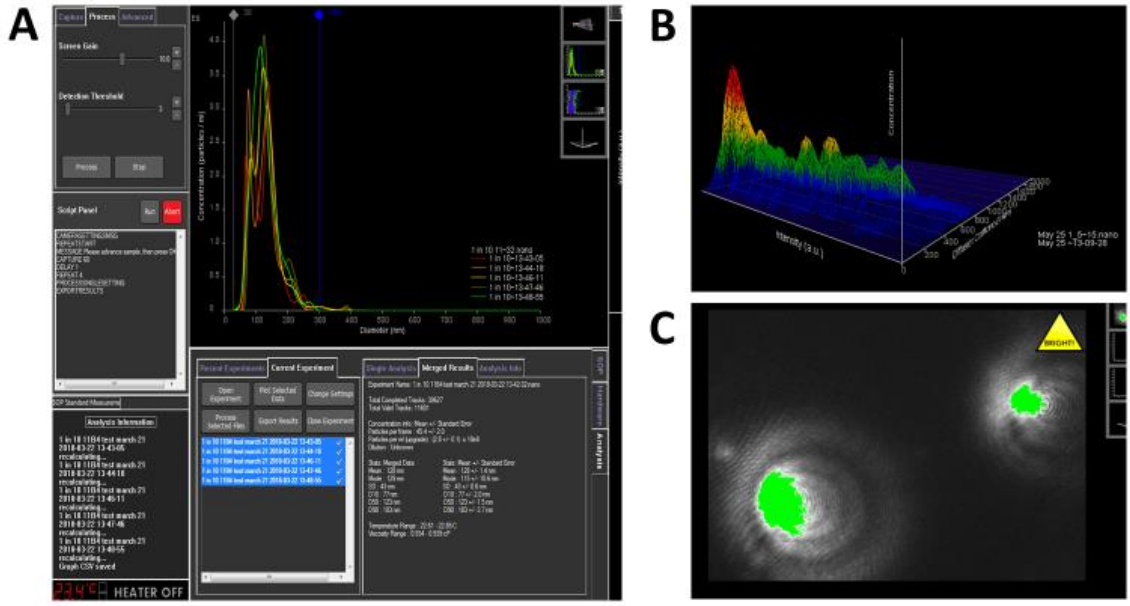


Figure 3-3 Analysis of EVs isolated from 1.1B4 cells using NanoSight NS300 Nanoparticle Tracking Analysis software. Representative image of the software programme during NTA (A) The coloured graphs indicate an individual 'frame' or measurement, 5 frames are run at 60 sec per frame and averaged to calculate the mean result. (B) Real-time measurement of the concentration (y-axis) and intensity (a.u.) (x-axis) of particles as they are illuminated by the laser beam. (C) Example of contaminants captured on camera found in unfiltered H_2O (i.e. tap water).

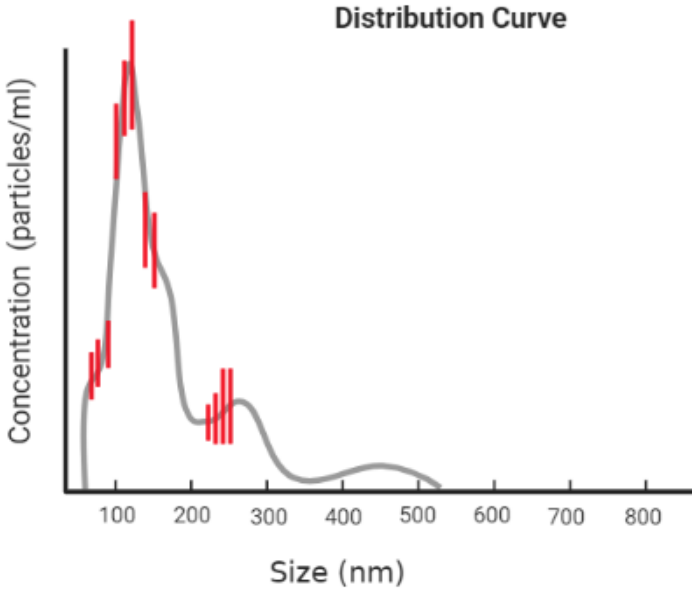


Figure 3-4 Representative graph of extracellular vesicle populations calculated using Nanoparticle Tracking Analysis (NTA). The graph represents the typical size distribution found within extracellular vesicle preparations. The black line represents the average frame captured over 5 frames, and the red lines indicate areas of high variance from the mean.

EV samples were diluted to reach an optimal range of particles per frame for NTA because either too few, or too many particles per frame reduces the accuracy of the measurements and the robustness of the results. Running samples below 30 particles per frame results in higher polydispersity (Figure 3-5) and this can be seen by the increased number of peaks on the graphs with <30 particles/frame. NanoSight Malvern technicians recommend that <44 particles/frame could affect the reproducibility of results, thus, samples were appropriately diluted to account for these hazards.

EVs from 1.1B4 cells were successfully isolated using the Thermo Fisher™ Total Exosome Isolation Kit and NTA indicated that the major subpopulations were within the accepted size range of exosomes (50-130 nm).

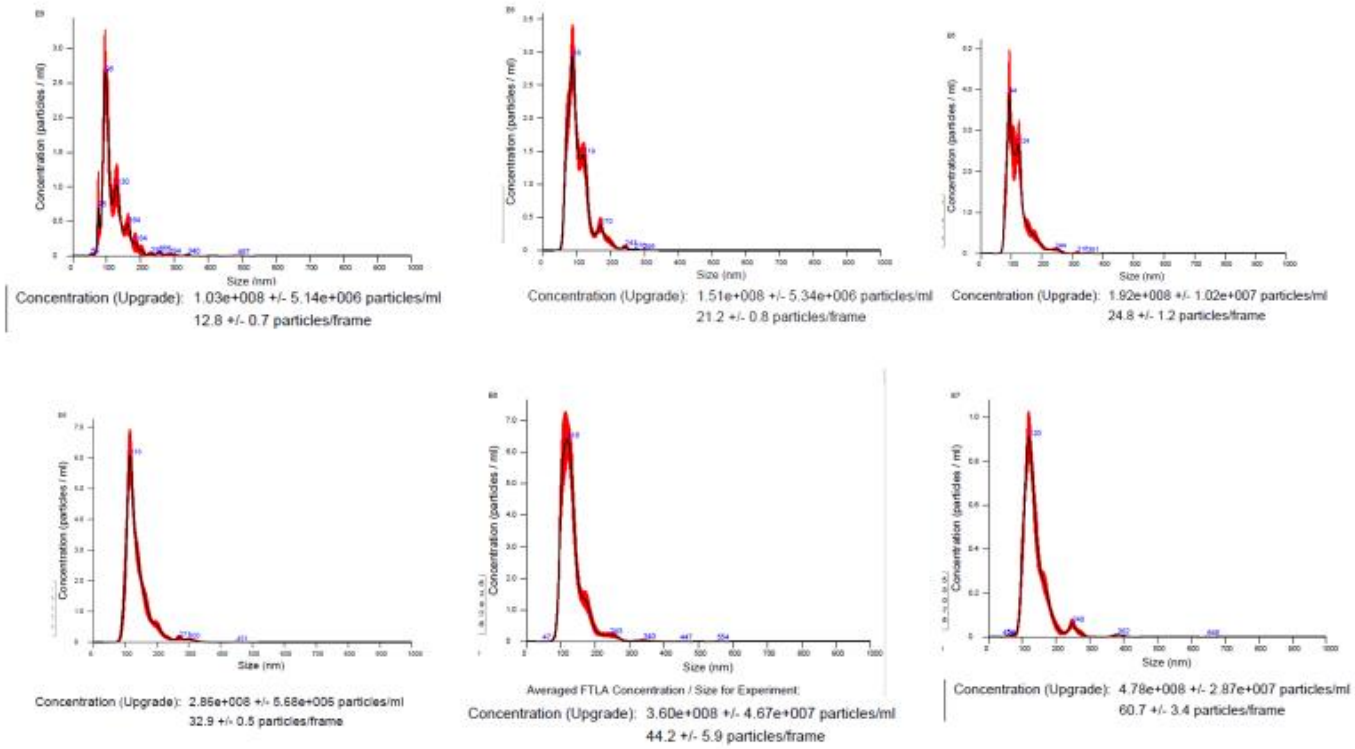


Figure 3-5 Nanoparticle Tracking Analysis of EVs isolated from 1.1B4 cells. Dilution factors with >44 particles/frame lead to more robust results. Series of graphs demonstrate that dilution of samples can affect outcomes and levels of polydispersity.

3.3.2 Characterising EVs using Western Blotting and EV Markers

After confirming that the Total Exosome Isolation Reagent successfully isolated EV samples from 1.1B4 culture supernatant, the next step was to confirm that these EVs carried recognised EV markers using Western Blotting techniques. There are several online resources available that document and collate molecular data on EVs (ex. ExoCarta, Vesiclepedia). Vesiclepedia (http://microvesicles.org/) provides a list of the top 100 proteins identified in EVs and has been compiled from a breadth of studies. The top 12 proteins from this list are provided in Table 3-5, but in addition to these proteins CD81 is also cited regularly as a key marker of EVs (Campos-Silva et al., 2019) and ranks 31st on the list.

Rank	Gene Symbol	Gene Name
1	PDCD6IP (ALIX)	Programmed cell death 6 interacting protein
2	GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
3	HSPA8	Heat Shock Protein 8
4	ACTB	Actin, beta
5	ANXA2	Annexin A2
6	CD9	CD9 antigen
7	PKM	Pyruvate kinase, muscle
8	HSP90AA1	Heat shock protein 90, alpha (cytosolic), class A member 1
9	ENO1	Enolase 1, (alpha)
10	ANXA5	Annexin 5
11	HSP90AB1	Heat shock protein 90 alpha (cytosolic), class B member 1
12	CD63	CD63 molecule

Table 3-5 The top 12 proteins identified in EVs from Vesiclepedia (http://microvesicles.org/).

There is no one specific marker for EVs but vesicles are highly enriched in tetraspanins CD9, CD63 and CD81, and these are widely referenced in published studies as markers of exosomes. CD9, CD63, and CD81 have a

broad tissue distribution and as such have been found in EVs from a multitude of tissue types (Andreu & Yanez-Mo, 2014).

Before isolating EVs, I chose a panel of antibodies against CD9, CD63 and CD81 (Table 3-2) and optimised methods for protein detection using cellular lysates from cell lines 1.1B4, PANC-1, EndoC-βH1, HeLa, HEK293 and SHSY5Y. A review of the Human Protein Atlas indicated that HeLa, HE293 and SHSY5Y cells express these tetraspanins and could thus act as positive controls for the pancreatic cell lines (Human Protein Atlas available from http://www.proteinatlas.org).

After extracting protein from the above cell lines, the samples were prepared for Western blotting and probed with anti-sera against HSP90 B1 [GRP94]. This antibody was chosen because it is present on exosomes and was previously found in EVs released from human islets (Hasilo et al., 2017). PANC-1, EndoC-βH1, HeLa, HEK293 and SHSY5Y cells were all found to express HSP90 B1 [GRP94] at the expected molecular weight of 90kDa (Figure 3-6). However, this protein was not detected in the 1.1B4 cell line.

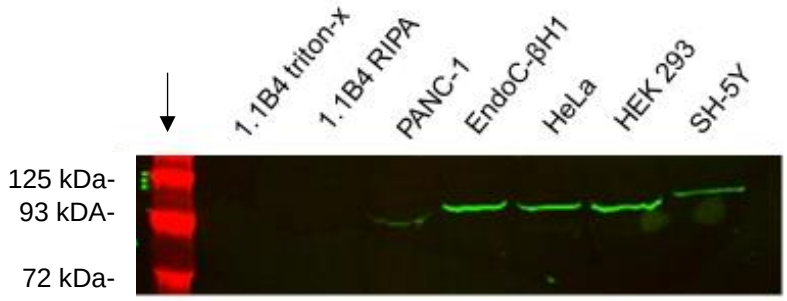


Figure 3-6 Whole cell testing for HSP90 [GRP94]. Tissue culture samples from 1.1B4, PANC-1, EndoC-βH1, HeLa, HEK 293 and SHSY5Y cells were harvested, prepared for fluorescent Western blotting and probed with anti-sera for HSP90 B1 [GRP94] and secondary antibody conjugated to DyLight™ 800 (green). Protein from 1.1B4 cells was extracted using triton-x and RIPA lysis buffers. 1.1B4 cells did not express the HSP90 GRP94 protein. 20 ug of protein was loaded for each sample. Red markers on the left (black arrow) indicate the protein ladder to calculate molecular weight for proteins shown in green. Representative example of n=3.

The 1.1B4 and EndoC-βH1 cell lines were both reported to physiologically resemble human β-cells so it was unusual that 1.1B4 cells did not stain positive for HSP90 B1 [GRP94]. I then proceeded to test an alternative antibody against HSP90, anti-HSP90 AC88 (Figure 3-7). Interestingly, all cell lines were found to test positive for HSP90 with the HSP90 AC88 antibody, and the nature of lysis buffer used on the 1.1B4 cells did not affect the results.

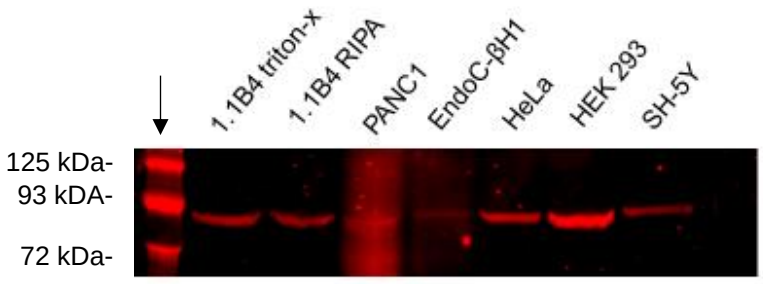


Figure 3-7 Whole cell testing for HSP90 [AC88]. Tissue culture samples from 1.1B4, PANC-1, EndoC-βH1, HeLa, HEK 293 and SHSY5Y cells were harvested, prepared for fluorescent Western blotting and probed with anti-sera for HSP90 AC88 and secondary antibody conjugated to DyLight™ 680 (red). All cell lines expressed the HSP90 AC88 protein. 20 ug of protein was loaded for each sample. Red markers on the left (black arrow) indicate the protein ladder to calculate molecular weight for proteins shown in red. Representative example of n=3.

I then proceeded to test the β-cell lines for the tetraspanin marker CD63 using an anti-CD63 antibody that recognised the LIMP antigen, a 30-60 kDa (smear) protein (Figure 3-8). As seen with the HSP90 B1[GRP94] antibody, all cell lines except 1.1B4 expressed the CD64 protein. Previous studies noted a smeared pattern for CD63 (40-65 kDa) from cells and exosomes using the clone TS63 (Oksvold et al, 2014) and this smear is likely due to glycosylation.

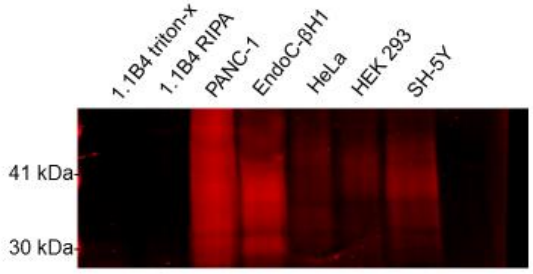


Figure 3-8 Whole cell testing for the CD63 protein. Tissue culture samples from 1.1B4, PANC-1, EndoC-βH1, HeLa, HEK 293 and SHSY5Y cells were harvested, prepared for Western blotting and probed with anti-sera for CD64 and secondary antibody conjugated to DyLight™ 680 (red). All cell types expressed CD64 except for 1.1B4 cells. 20 ug of protein was loaded for each sample. Representative example of n=2.

3.3.3 Testing EVs from 1.1B4 cells for EV markers

After confirming that the clonal β-cell lines expressed two known EV markers, I proceeded to isolate EVs from 1.1B4, PANC-1 and HeLa cells. To ensure that optimal protein extraction methods were used, I tested three different lysis buffers to extract protein from 1.1B4 cells. These three lysis buffers have been used regularly for studies in our laboratory. Triton-X and RIPA are commonly used lysis buffers that differ in their ability to solubilize proteins. RIPA buffer contains an ionic detergent and is considered to produce a higher protein yield due to its harsh nature. It will disrupt protein-protein interactions and is generally used when extracting membrane bound proteins and proteins found within the nucleus or mitochondria. Triton-X100 is a milder detergent and generally used for extracting protein found within the whole cell (Ji, 2010).

After lysis, EVs were isolated by UC at 100,000 x g for 70 min and protein was extracted as described in the methods (3.2.7 and 3.2.8). To determine if EV marker CD9 was present in the isolated EV samples, antibody probing was conducted using Western blotting and a human anti-CD9 antibody (Figure 3-8). HeLa EVs were used as a positive control after confirmation of CD9 expression was found on the Human Protein Atlas (Human Protein Atlas available from http://www.proteinatlas.org). EVs isolated from PANC-1 and HeLa cells were found to express tetraspanin CD9, while all EV samples from the 1.1B4 cell line were negative for CD9 expression. For this experiment, the milder lysis buffer (Triton-X100) was found to be effective for protein extraction of EVs from PANC-1 and HeLa cells. This experiment also demonstrated that the nature of the lysis buffer used not affect the results for 1.1B4 cells. The membranes were re-probed with an antibody recognising second tetraspanin, CD81, but similar results were observed (Figure 3-9).

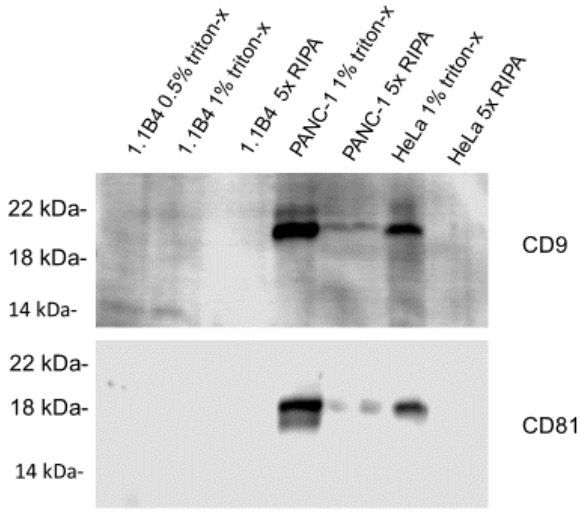


Figure 3-9 Whole cell testing for the CD9 and CD81 protein. EV samples from 1.1B4, PANC-1, and HeLa cells were collected at 100,000 x g. Protein was harvested and prepared for chemiluminescent Western blotting and probed with anti-sera for CD9 and CD81, and HRP-tagged secondary antibody. Representative example of n=3.

To determine if protein conformation and folding states affected efficacy of antibody recognition and binding, I prepared EV protein samples for Gel Electrophoresis using reducing and non-reducing conditions. Reducing conditions involve the use of β-mercaptoethanol and heat to reduce the number of disulphide bridges in proteins, however non-reducing conditions are recommended for studying tetraspanins to preserve protein conformation. EVs from 1.1B4 cells were isolated using UC at 100,000 x g and prepared for Western blotting before probing with anti-sera against CD9, CD63 and CD81. Since all three human antibodies failed to recognise their respective targets, I determined that protein conformation did not affect the results (Figure 3-10).

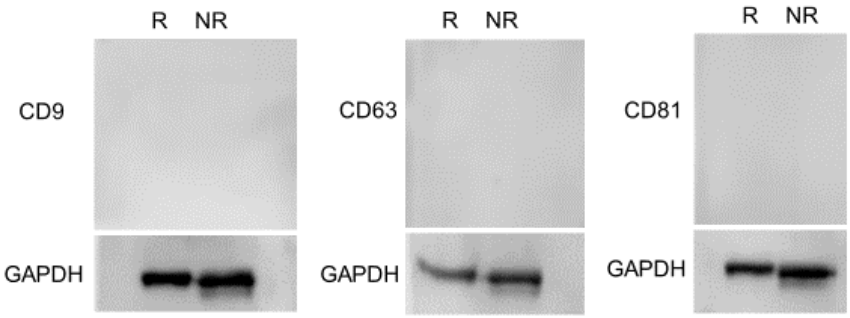


Figure 3-10 Sample preparation with non-reducing conditions does not affect detection of human tetraspanins on EVs isolated from 1.1B4 cells.

EVs were isolated by UC at 100,000 x g and protein samples were prepared for gel electrophoresis under reducing (R) or non-reducing conditions (NR) as described in methods section 3.2.11 . Samples were then prepared for chemiluminescent Western blotting and probed with anti-sera for CD9, and HRP-tagged secondary antibody. GAPDH was detected as a loading control. Representative example of n=3.

3.3.4 Flow cytometric analysis of EVs isolated with magnetic beads from 1.1B4 cells

In conjunction with the above experiments, EVs were collected from 1.1B4 cells using the Total Exosome Isolation Reagent Kit. To determine if the isolated 1.1B4 EV samples expressed CD9 on their surface, I used the Human CD9 Flow Detection method as outlined in the methods (section 3.2.16). The Human CD9 Flow Detection method is intended for isolation of CD9 positive EVs from isolated EV samples. It uses Dynabeads^{TM} (Cat. No. 10620D), which are superparamagnetic polystyrene beads (2.7 um diameter) coated with a primary monoclonal antibody specific for the CD9 membrane antigen expressed on most human exosomes. The magnetic beads are incubated with the EV samples overnight and isolated CD9+ EVs are magnetically separated for further analysis using flow cytometry.

1.1B4 EV samples were bound to beads and stained with fluorescent anti-human CD9 antibody as described above and processed for flow cytometry analysis using BD FACS Aria III. An isotype control was also prepared. CD9 was not detected on the surface of EVs even after a 48 hr incubation combining the CD+ Magnetic beads and EVs (Figure 3-11).

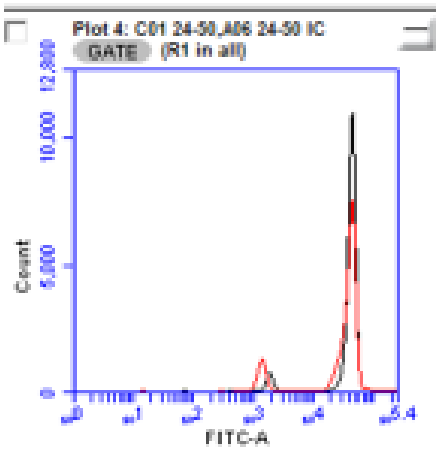


Figure 3-11 Flow cytometric analysis of exosomes isolated with magnetic beads. 1.1B4 derived EVs were immunoisolated from pre-enriched EV sample using magnetic beads coated with anti-human CD9. Antibodies against human CD9 were treated with EVs followed by flow cytometric analysis and presented as Median Fluoresence Intensity (MFI); red line represents MFI of EV samples, black line represents control. Calculated using n=3 samples.

3.3.5 1.1B4 cells are of rat origin and not human

My colleague, Dr. Jessica Chaffey, led a full-scale investigation into the characteristics of 1.1B4 cells (Chaffey et al. 2021) and concluded that the 1.1B4 cell line is composed of heterogenous populations of cells arising from both humans and rodents. Our team observed that successive passages of these cells led to diminution in the sub-population of human cells and a resultant expansion of the rodent cells.

When the cells initially became available for distribution, DNA barcoding (Nims et al., 2010) was conducted by the European Collection of Authenticated Cell Cultures (ECACC) to verify the cells. However, DNA barcoding is an assay that amplifies mitochondrial DNA sequences to determine the species of the cell line and cannot be used to assess if DNA originates from two different species. The test can detect human nuclear DNA but not the presence of contaminating DNA from a different species. Therefore, in the instance of 1.1B4s, verification by DNA barcoding was insufficient to appropriately identify the cells. After being notified of the concerns around 1.1B4 cells, ECACC confirmed the presence of human and rat nuclear DNA sequences in the cells.

The product from electrofusion of cell lines is usually a heterokaryotic line, which has nuclear DNA representing both the recipient and donor cell line. What may help to explain the case of the1.1B4 line, is a report in the literature that proposed hybrid cells fused from two different species will initially contain cytoplasm and mitochondria from both species but that the mitochondria from one species can quickly dominate (Attardi and Attardi, 1972).

These findings were confirmed across a number of archived stocks, including those held by ECACC. The conclusion drawn was that the 1.1B4 cells in our collection did not represent an authentic human beta cell line and extreme

caution should be used when considering the use of these cells as a model system for β-cell biology. These findings have resulted in the withdrawal of 1.1B4 (and other related cell lines) from ECACC.

This discovery impacted the direction of my research and I looked to other pancreatic cell models to complete my studies. I proceeded with PANC-1 and EndoC-βH1 cells which significantly increased the length of time of each experiment because EndoC-βH1 cells take substantially longer to propagate in the laboratory. In some instances, a confluent T75 flask of EndoC-βH1 cells could take up to 4 weeks to propagate.

3.3.6 Comparing glucose concentrations on exosome polydispersity in PANC-1 and EndoC-βH1 cells

Previous studies using trophoblast cells have suggested that exosome release may be affected by glucose concentrations (Rice et al., 2015) which could potentially have significance for pancreatic cell models. To date, there have been no studies exploring the effect of glucose on EV release from pancreatic cells, therefore I tested whether varying glucose levels affected polydispersity of EVs from PANC-1 and EndoC-βH1 cells. Polydispersity can be described as representing the size distribution within a sample, or the range of particle diameters. PANC-1 cells are generally unresponsive to glucose, but under certain conditions have been noted to express a differentiated phenotype in which cells exhibit response to glucose (Hiram-Bab et al., 2012). Whereas EndoC-βH1 cells change their rate of secretion of insulin in response to altered glucose conditions (Tsonkova et al., 2018).

PANC-1 and EndoC-βH1 cells were incubated with 5 mM or 25 mM of D-glucose. In addition, since the osmolality affects cell morphology and functions, L-glucose was used as an osmotic control to determine the true effect of

changing D-glucose concentration on EV release (Huang and Xiong, 2015). EVs were isolated by ultracentrifugation at 10,000 x g for 30 min or 100,000 x g for 70 min. Samples were then analysed for polydispersity using NTA (Figure 3-12).

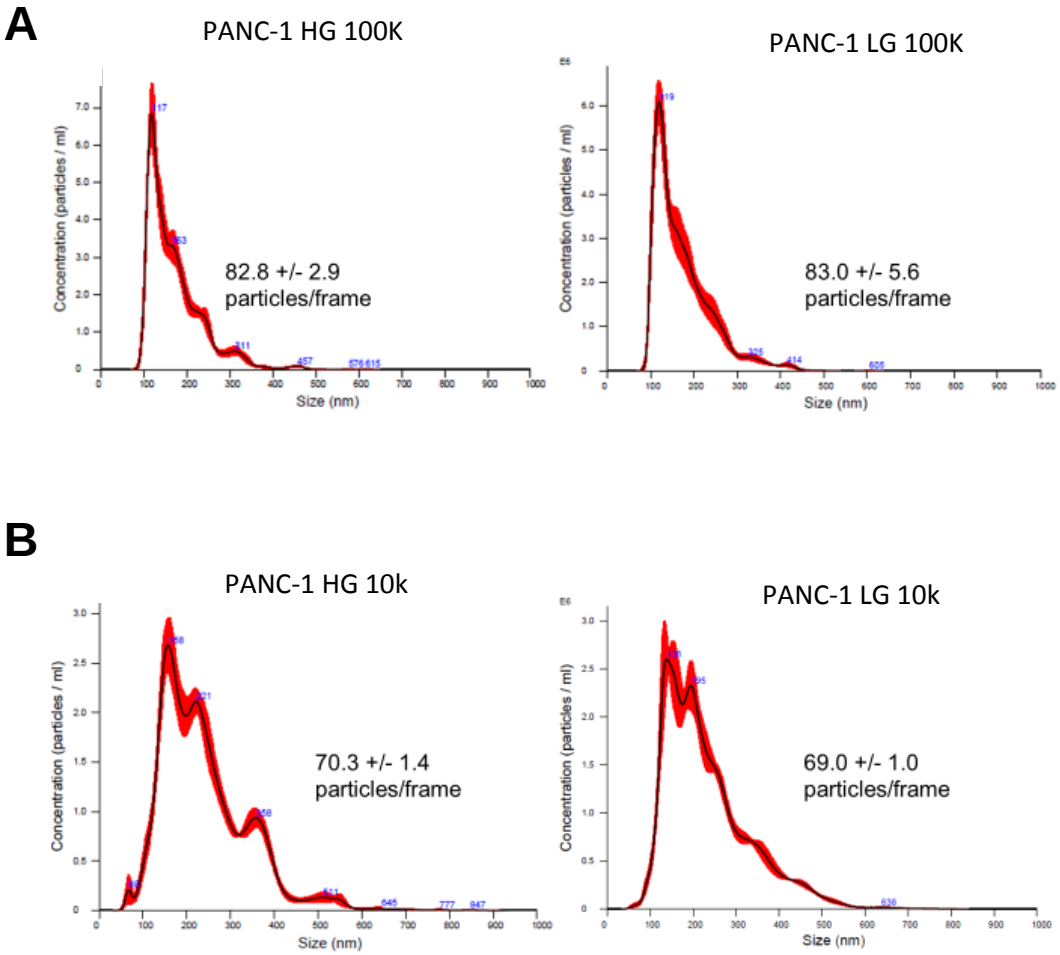


Figure 3-12 EVs collected from PANC-1 cells cultured in high (HG) and low (LG) glucose medium as measured by Nanoparticle Tracking Analysis. (A) EVs isolated from centrifugation at 100,000 x g (B) EVs isolation from centrifugation at 10,000 x g. Representative example of n=3.

EVs isolated from PANC-1 cells had significant polydispersity in samples collected at 10,000 x g compared to 100,000 x g. Polydispersity can be observed by the shape and number of peaks in each graph. Table 3-6 describes the peak sizes observed in each condition. EVs cultured in high glucose medium (25 mM) and collected at 10,000 x g, had distinct peaks corresponding to EV diameter sizes of 158 nm, 221 nm and 358 nm. EVs cultured in low glucose medium (5 mM) and collected at 10,000 x g had more uniform distribution with subgroups concentrated between 100-200 nm, but polydispersity was also present. There were small subpopulations of EVs sized between 500-600 nm.

Condition	Highest peak size (nm)
PANC-1 HG 100K	117
PANC-1 LG 100k	119
PANC-1 HG 10K	158
PANC-1 LG 10K	130

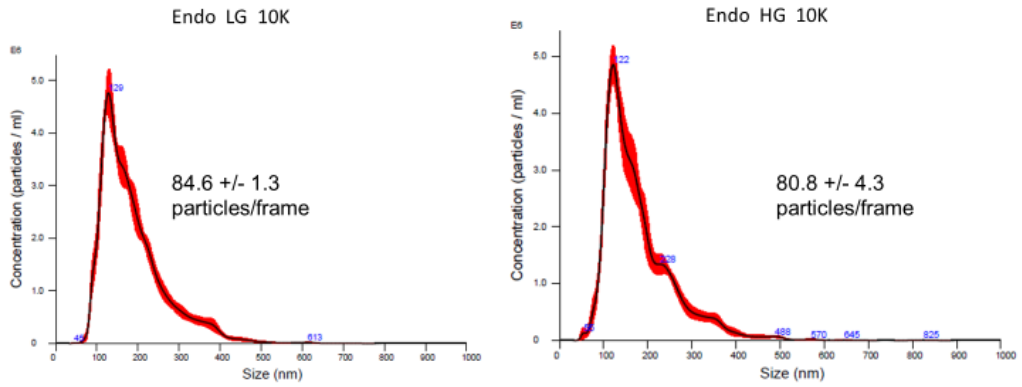
Table 3-6 Peak sizes as calculated by Nanoparticle Tracking Analysis for PANC-1 EVs collected under high glucose (HG) and low glucose (LG) conditions and centrifuged at 100,000 x g or 10,000 x g.

PANC-1 EV samples collected at 100,000 x g contained a large subgroup of vesicles around 117 - 119 nm in EV diameter size, which indicates large populations of exosomes. The subpopulations of EVs at 500-600 nm are much smaller in size suggesting those larger sized EVs were removed through the ultracentrifugation process.

EVs isolated from EndoC-βH1 cells and collected at 10,000 x g in both high and low glucose medium were similar in population characteristics, both having

large sub-populations of vesicles around 125 nm in size (Figure 3-13). Table 3-7 describes the peak sizes observed in each condition. Compared to PANC-1 cells, the overall population of EVs were more uniform with less polydispersity.

A



B

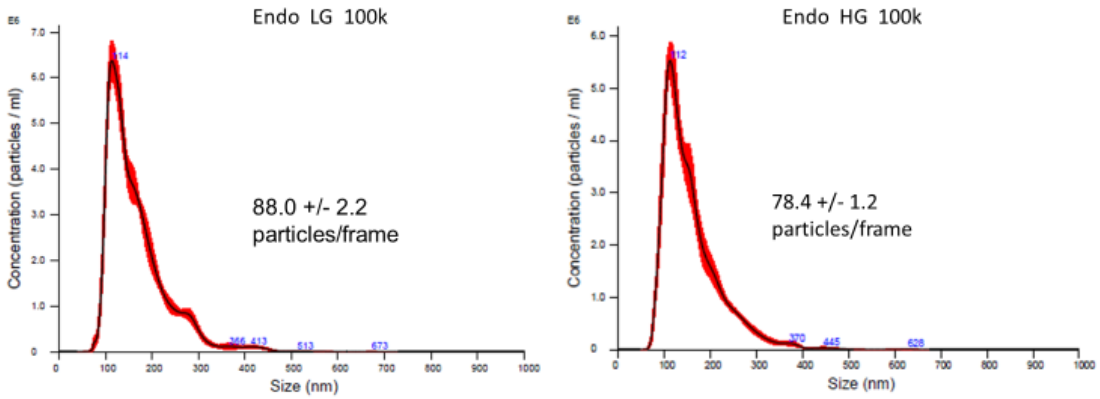


Figure 3-13 EVs collected from EndoC-βH1 cells cultured in high (HG) and low (LG) glucose medium as measured by Nanoparticle Tracking Analysis. (A) EVs isolated from centrifugation at 10,000 x g. (B) EVs isolation from centrifugation at 100,000 x g. Representative example of n=3.

Condition	Highest peak size (nm)
EndoC-βH1 HG 100K	112
EndoC-βH1 LG 100K	114
EndoC-βH1 HG 10K	122
EndoC-βH1 LG 10K	129

Table 3-7 Peak sizes as calculated by Nanoparticle Tracking Analysis for EndoC-βH1 EVs collected under high glucose (HG) and low glucose (LG) conditions and centrifuged at 100,000 x g or 10,000 x g.

3.3.7 Exosome inhibitors and PANC-1 cells

An additional method to conclusively verify that exosomes were in the samples of EVs collected was to block pathways involved in exosome generation and release. GW4869 is a potent neutral sphingomyelinase inhibitor that interferes with exosome secretion and vesicle trafficking (Catalano and O'Driscoll, 2019, Datta et al., 2018) However, I encountered several difficulties when using this drug and I was not able to get the drug into solution with DMSO, despite following the technical data sheets as supplied by Sigma (Sigma; cat. D1692). As a result, I did not observe GW4869 inhibition of exosome release as shown in Figure 3-14A. Large populations of vesicles around 50-130 nm in size were still observed following NTA.

A second drug was also used to inhibit exosome generation and release but this also failed to enter into solution with DMSO. Various concentrations of N-SMase Spiroepoxide (Sigma) were unable to prevent the release of vesicles ranging the sizes of 30-150 nm (Figure 3-14B). Even visually on the NTA camera, there did not appear to be a reduction in the number of vesicles illuminated by the laser beam. With the difficulties encountered in using the drugs, it was not possible to conclude if the actions of the drugs were effective in PANC-1 cells. While these drugs are commonly referenced to block exosome release (Catalano and O'Driscoll, 2019), some studies have found they are ineffective on certain cell types (Phuyal et al., 2014).

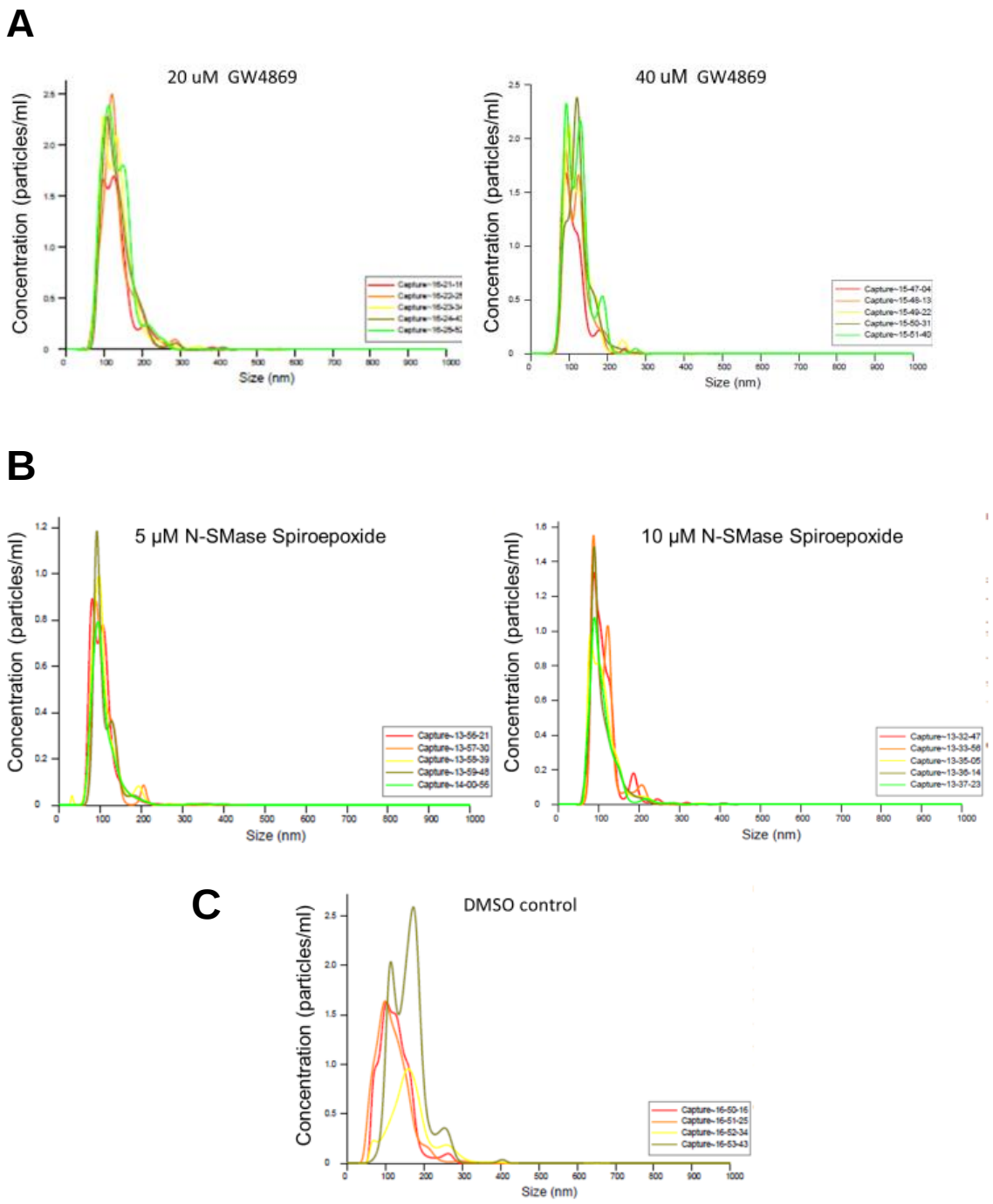


Figure 3-14 Exosome drug inhibitors GW4869 and N-SMase Spiroepoxide did not block production of vesicles 30-150 nm in size as measured by Nanoparticle Tracking Analysis (NTA). The different colours in each graph represent a separate frame or count as opposed to the mean value (n=5). (A) NTA values of vesicles released after treatment with GW4869 (B) NTA values of vesicles released after treatment with N-SMase Spiroepoxide (C) DMSO control. Representative example of n=2.

3.3.8 Characterising EVs derived from PANC-1 and EndoC-βH1

After the discovery that 1.1B4 cell cultures were contaminated with cells of rodent origin, I focused on characterising EVs from PANC-1 and EndoC-βH1 cells. During the analysis of the 1.1B4 cell line, we did confirm that the EndoC -βH1 and PANC-1 cell lines were of human origin using a vomeronasal species specific PCR (Chaffey et al., 2021). Using the high and low glucose conditions outlined in the above section, I collected the EVs from these cells at 100,000 x g, prepared them for Western blotting and probed with anti-sera against CD9 and ALIX (Figure 3-15A). ALIX, also known as Programmed cell death 6 interacting protein, is the most cited protein found in exosomes according to Vesiclepedia. Interestingly, there were two protein bands seen around 24 kDa for CD9 and the lower band may be a palmitoylated form of CD9 as seen elsewhere in the literature (Espenel et al., 2008). EVs from PANC-1 and EndoC-βH1 cells both expressed CD9 and EVs from PANC-1 also expressed ALIX. The EV samples isolated from PANC-1 also stained positively for CD81 (Figure 3-15B). Further tests on EVs isolated from EndoC-βH1 cells in low glucose medium confirmed that EVs isolated from EndoC-βH1 cells express CD9 and ALIX (Figure 3-15C). The pattern of CD9 expression could indicate palmitoylated forms.

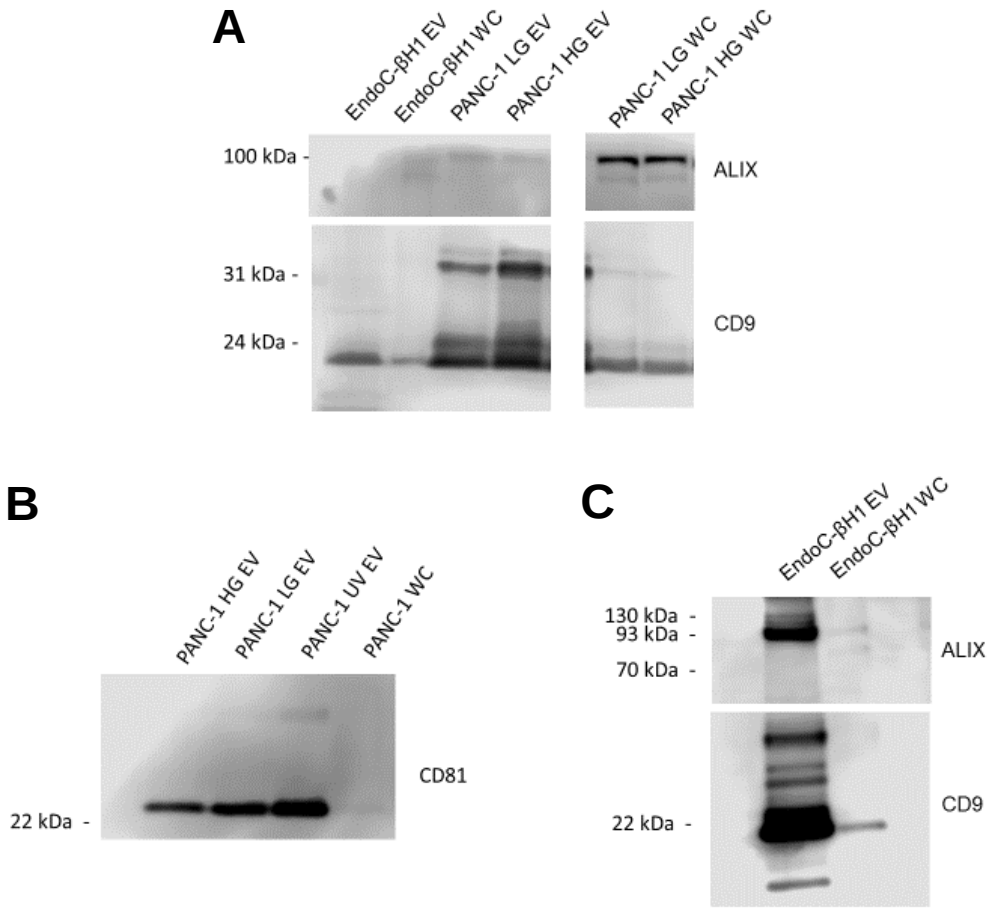


Figure 3-15 Typical exosome markers are present on EVs isolated from pancreatic cells. (A) PANC-1 and EndoC-βH1 cells were propagated in high and low glucose conditions and EVs were isolated using UC at 100,000 x g. EV samples were prepared for chemiluminescent Western blotting and probed with anti-sera for ALIX and CD9. (EV= extracellular vesicle; LG = Low Glucose medium; HG = High Glucose medium; UV = Ultraviolet radiation treatment on cells; WC = whole cell lysate) (B) EVs isolated from PANC-1 cells also express CD81 (C) EVs isolated from EndoC-βH1 cells express CD9 and ALIX. Representative example of n=3.

3.3.9 Inducing expression of PD-L1 on the surface of EVs with IFNs

Chen et al. (2018) demonstrated that PD-L1 could be expressed on the surface of EVs released from melanoma cells and that stimulation with IFN-γ increased the amount of extracellular PD-L1. Moreover, PD-L1 on EVs could suppress the function of CD8 T cells and facilitate tumour growth. Therefore, I sought to explore if IFNs could also induce the expression of PD-L1 on EVs isolated from pancreatic cells. I wanted to demonstrate 1) that PD-L1 was present on PANC-1 and EndoC-βH1 cells and 2) when stimulated with IFNs, these cells can release EVs with PD-L1 on the surface.

PANC-1 cells were treated with IFNs (IFN-γ and IFN-α) and EVs were collected using UC at 100,000 x g. Samples were prepared for Western blotting and probed with anti-sera for PD-L1 and CD9. PD-L1 was found to be expressed in EV samples with and without IFN treatment (Figure 3-16A). EndoC-βH1 cells were also treated with IFN-γ and IFN-α and isolated EVs also expressed PD-L1 (Figure 3-16B). Experiments with EndoC-βH1 cells were particularly difficult to achieve because they propagate very slowly in the laboratory (months) and prefer to grow in small tissue culture flasks (T25 holding 5 mL of media). EndoC-βH1 cells were treated with IFN-α and EVs were collected at 10,000 x g and 100,000 x g to determine if EV subpopulations affected PD-L1 expression. Both samples were found to express PD-L1 which indicates exosomes and microvesicles express PD-L1 (Figure 3-16C). Whole cell lysates from human islets and pancreatic cells were used as positive controls since they were previously shown to express PD-L1 (**Chapter two)** (Colli et al., 2018). Probing for PD-L1 was challenging and the results often showed smeared protein (Figure 3-16B). EVs generally produce very small quantities of detectable protein, which is why large volumes (>60 mL) of conditioned media are required

to analyse EVs. During the probing stages (following Western blotting) a high sensitivity ECL substrate (Abcam) was required because it could detect proteins with 4.6 pg - 4.7 ng of protein per band. The low level of protein available also made it difficult to re-probe membranes for additional proteins as the they would get removed with the stripping buffer.

To verify if PD-L1 expression was found in different subtypes of EVs, I treated EndoC-βH1 with IFN-α and collected the EVs at 10,000 x g and 100,000 x g. PD-L1 was found to be expressed in all samples, irrespective of UC speed. EV marker CD63 was also found to be expressed on these EVs. Nanosight NTA confirmed that the EVs collected at 100,000 x g contained major subgroups of vesicles between 50-130 nm in size, thus verifying the presence of exosomes.

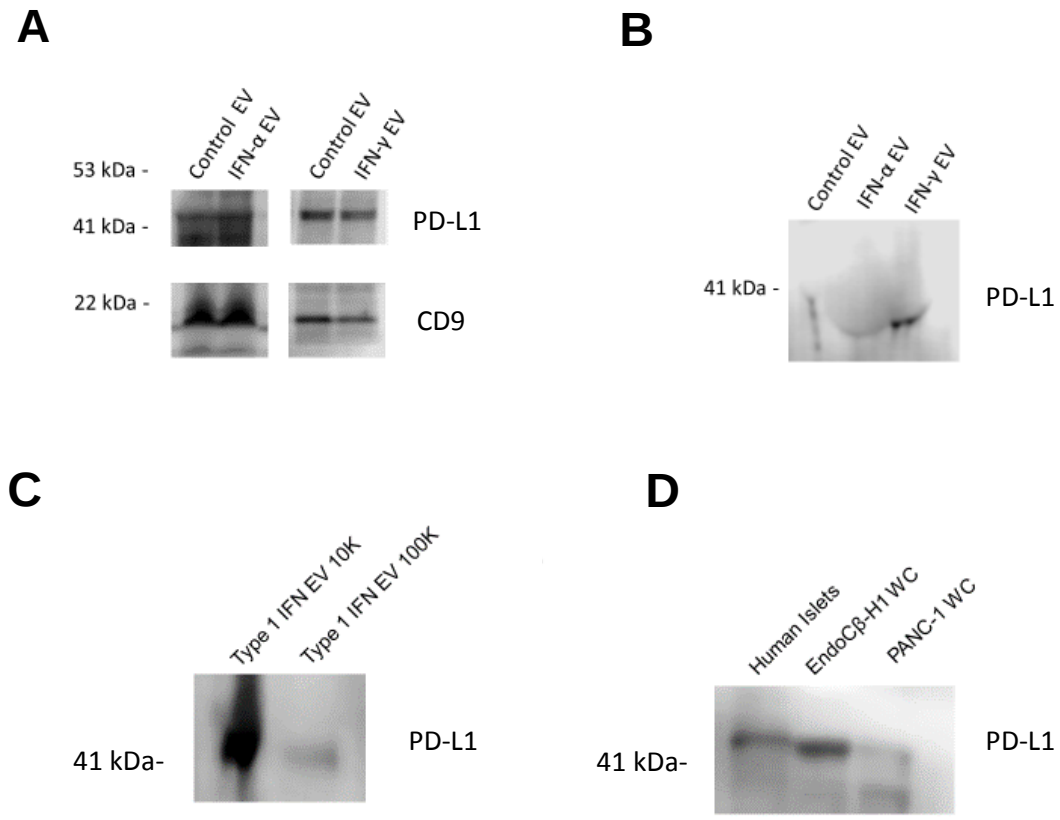


Figure 3-16 EVs isolated from pancreatic cells express PD-L1 (predicted molecular weight 45-70 kDa). (A) EVs from IFN treated (IFN-α and IFN-γ) PANC-1 were isolated using UC at 100,000 x g, prepared for chemiluminescent Western blotting and probed with anti-sera for PD-L1and CD9. (B) EVs from IFN treated (IFN-α and IFN-γ) EndoC-βH1 were isolated using UC at 100,000 x g, prepared for Western blotting and probed with anti-sera for PD-L1. Smearing was frequently observed. (C) EVs isolated from IFN treated (IFN-α) EndoC-βH1 using UC at 10,000 x g and 100,000 x g expressed PD-L1. (D) Whole cell (WC) lysates of PANC-1, EndoC-βH1 and human islets expressed PD-L1. Control was no IFN treatment. Representative examples of n=3.

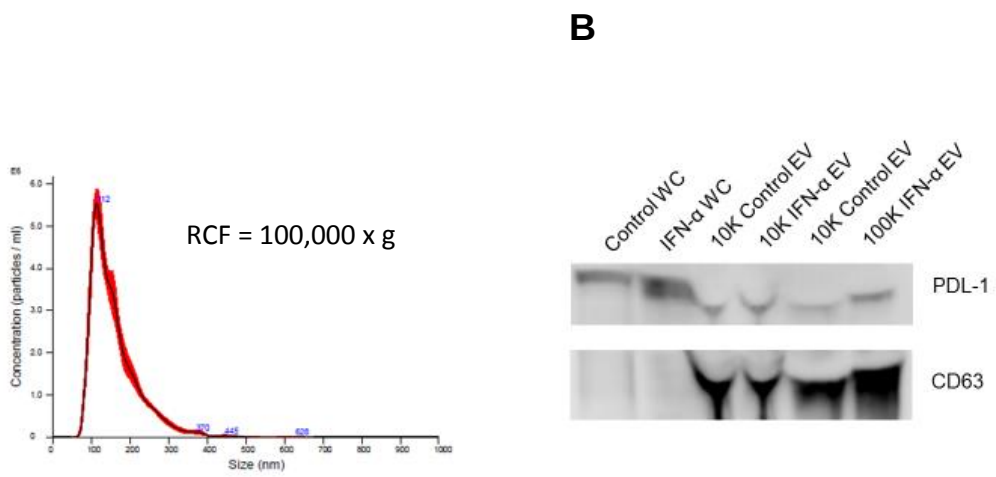
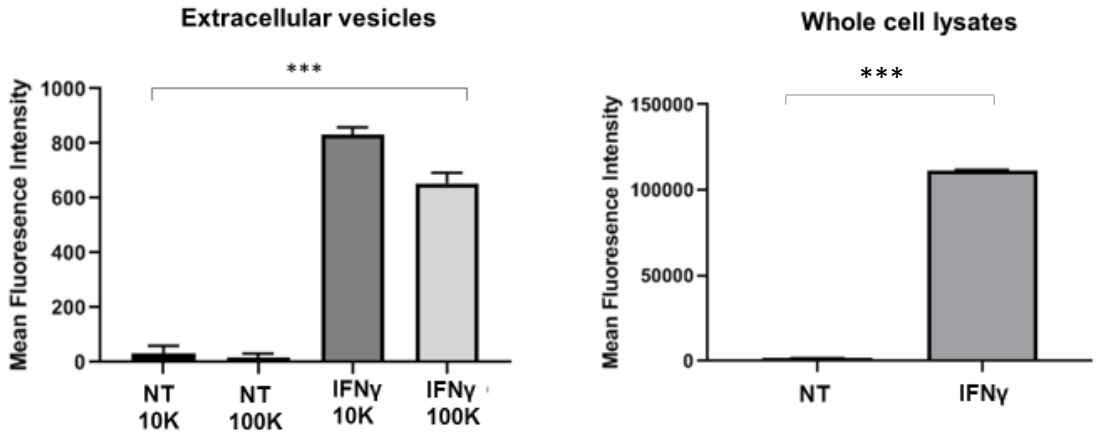


Figure 3-17 PD-L1 is expressed on small EVs (exosomes collected at 100,000 x g) and larger EVs (collected at 10,000 x g). (A) Representative graph of small EV population (exosomes) from IFN treated EndoC-βH1 cells collected at 100,000 x g and analysed using Nanoparticle Tracking Analysis (representative example of n=3) (B) EVs from IFN treated or control EndoC-βH1 cells were collected at 10,000 x g and 100,000 x g and prepared for chemiluminescent Western blotting. Antibody probing was conducted using anti-PD-L1 and anti-CD63 antibodies. Whole cell (WC); control (no IFN treatment); 10 K (10,000 x g); 100 K (100,000 x g). Representative example of n=3.

3.3.10 Flow Cytometry Analysis of PD-L1 on the surface of EVs.

Due to the challenges in Western blotting as explained above, other methods were designed to determine if IFNs induced higher expression of PD-L1 on the surface of EVs. I designed a method using Flow Cytometry and superparamagnetic polystyrene beads (2.7 um diameter) coated with a primary monoclonal antibody specific for CD9. As demonstrated in the previous chapter and Colli et al. (2018), IFN-γ was found to induce higher levels of PD-L1 expression, therefore, I used IFN-γ for the following experiments. EVs were isolated from IFN and control treated EndoC-βH1 and PANC-1 cells using UC at 10,000 x g and 100,000 x g. Samples were then incubated with the superparamagnetic polystyrene beads before fluorescently tagged antibody detection with CD9 (as described in methods section 3.2.17). Flow cytometry analysis demonstrated that IFN-γ significantly induced expression of PD-L1 on the surface of EVs from EndoC-βH1 but not PANC-1 cells (Figure 3-18).

A


Extracellular vesicles
***
Mean Fluoresence Intensity
1000
800
600
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200
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NT 10K
NT 100K
IFNγ 10K
IFNγ 100K
Whole cell lysates
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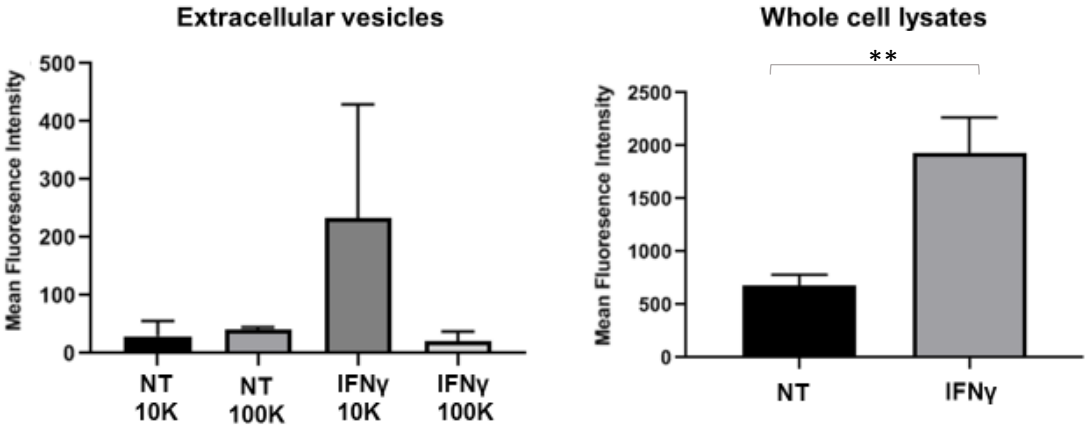
Extracellular vesicles
Mean Fluoresence Intensity
500
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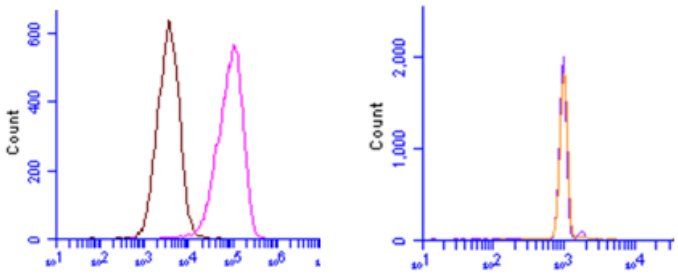


Figure 3-18 IFN-γ upregulates the cell surface expression of PD-L1 on EVs isolated from EndoCβ-H1 cells but not PANC-1 cells. (A) EVs isolated from IFN treated EndoCβ-H1 cells using UC at 10,000 x g and 100,000 x g showed significant upregulation of PD-L1 expression compared to control (NT= No IFN Treatment). Right image shows whole cell lysates as control for EV samples. Expression of PD-L1 was quantified using mean fluorescence analysis (MFI) .(***p<0.0001) significance was determined by one-way ANOVA (10K = 10,000 x g; 100k = 100,000 x g) (left image) or unpaired t test (right image). Error bars indicate SD (B) EVs isolated from IFN treated PANC-1 cells (left image) did not significantly express PD-L1 despite significance observed in whole cell lysate (right image). Expression of PD-L1 was quantified using mean fluorescence analysis (MFI). (**p<0.005) significance was determined by one-way ANOVA (left image) and unpaired t test (right image). Error bars indicate SD (C) PD-L1 cell surface expression was quantified by flow cytometry analysis at baseline or after exposure to IFN-γ. Left histogram represents PD-L1 surface expression analysis from EndoCβ-H1 cells (brown; baseline, pink; IFN-γ) and right histogram represents PD-L1 surface expression analysis from PANC-1 cells (yellow; baseline, red; IFN-γ). Representative examples of n=3 (each n represents 3 replicates).

3.4 Discussion

This chapter demonstrated that EVs of varying sizes can be isolated from pancreatic cell lines using a UC method. As predicted in section 3.1.3, UC at 100,000 x g and 10,000 x g produced EV samples with varying levels polydispersity but generally 10,000 x g resulted in the isolation of larger EVs. Propagating PANC-1 and EndoC-βH1 cells in varying concentrations of glucose (HG vs LG) demonstrated that glucose can influence the polydispersity of isolated EVs, particularly for PANC-1 cells. Recognised markers of exosomes were confirmed to be present on EVs isolated from pancreatic cells, as well as the expression of PD-L1. Additionally, testing of human antibodies on 1.1B4 cell lysates and the subsequent absence of protein recognition further supported evidence of a non-human origin.

3.4.1 1.1B4 cells and their true host origin

The discovery of the true origin of 1.1B4 cells brought clarity to the results observed in sections 3.3.2 and 3.3.3. Since 1.1B4 cells were believed to be human, the antibodies chosen for these experiments were known to react with human proteins. However, commercial antibodies will often cross-react with several species so the antibodies that recognised rat peptides were successful in staining for EV markers in 1.1B4 cells (example, HSP90 [AC88]). However, the antibodies that reacted exclusively with human (ex. CD9, CD81, and CD63) did not recognise their peptide targets in protein samples from 1.1B4 cells. This was further demonstrated by the absence of CD9 on EVs from 1.1B4 cells using flow cytometry analysis and magnetic beads with human anti-CD9 antibody.

3.4.2 Pancreatic cells release EVs and carry classic EV markers

Exosomes and EVs were successfully isolated from pancreatic cells and carried the typical EV markers. NTA data indicated EVs of varying sizes between <100

nm and >300 nm were isolated using the UC method. Samples isolated at 10,000 x g showed higher polydispersity and larger vesicle populations. Interestingly, glucose concentration did not substantially alter the EV subgroups released from EndoC-βH1 cells, but low glucose concentrations caused higher polydispersity in PANC-1 EVs collected at 10,000 x g. This is interesting because it may relate to the mechanisms of glucose induced insulin secretion as outlined in the introduction (1.5.3). β-cells do not have membrane-bound glucose receptors, so glucose enters the β-cell through facilitated diffusion via insulin-independent glucose transporters (GLUT-1 and GLUT-4 are known to be present in EndoC-βH1 cells) (De Vos et al., 1995, Richardson et al., 2007, Tsonkova et al., 2018). After entering the cell, glucose triggers metabolic pathways to induce insulin secretion. In brief, glucose is metabolised through the process of glycolysis, beginning with glucokinase (or hexokinase in other cell types) catalysing the phosphorylation of glucose to glucose – 6 – phosphate (Middleton, 1990). Compared to other cell types, higher concentrations of glucose are needed to trigger a response from glucokinase in β-cells because insulin is restricted for release during conditions when glucose levels are elevated beyond basal levels. This acts as a control mechanism for the release of insulin. Like insulin release, EV release from EndoC-βH1 cells may be more tightly regulated in response to glucose than PANC-1 cells, which do not release insulin. Interestingly, human β-cells have previously be found to release proinsulin within exosomes, but this study did not analyse the effect of glucose on exosome release (Cianciaruso et al., 2017).

It is incredibly difficult to isolate pure exosome populations from EVs subpopulations of a similar size range (100 nm to 200 nm in diameter size) but researchers have proceeded with describing exosomes so long as they

demonstrate NTA analysis, and show CD9, CD81, CD63 markers are present. As such, I conclude that my studies did indeed capture and analyse exosomes from pancreatic cells. Exosome markers CD9, CD63, CD81, ALIX and HSP90 were all present in EVs from PANC-1 and EndoC-βH1 cells.

Research on the significance of CD9 expression on β-cells is still in an early phase but one study found that β-cells could be categorized into four antigenically distinct subtypes, distinguished by differential expression of ST8SIA1 and CD9 (Dorrell et al., 2016). Another found that CD9 is a cell-surface marker for negative enrichment of glucose-responsive β-like cells differentiated from human pluripotent stem cells (Li et al., 2020a). The differential expression of CD9 across β-cells could have implications for researching exosomes in T1D. If expression of CD9 can distinguish the subtypes of β-cell, then analysis could be undertaken to determine if exosome cargo such as autoantigens, proinsulin, cholesterol, cytokines, proteins and miRNA altered depending on the subtype. Accompanied by microscopy and *in vivo* experiments, the loss of particular subtypes may affect disease progression, particularly if exosome release is altered. The theory that some β-cell subtypes may carry different cargo stems from researchers showing that the β-cell population *in situ* is operationally heterogeneous. Additionally, the same researchers have also proposed that hub cells exist within islets that not only have different molecular properties but also coordinate islet responses to glucose (Johnston et al., 2016).

3.4.3 PD-L1 is carried on the surface of EVs released from pancreatic cells and is upregulated by IFN stimulation

This chapter demonstrated that PD-L1 protein expression was present in EVs isolated from pancreatic cells through Western blotting. PD-L1 expression was

accompanied with typical exosome markers, which confirmed the presence of exosomes together with NTA analysis. PD-L1 expression was also confirmed to be present on the cell surface of EVs, and in keeping with findings from the previous chapter, was upregulated in the presence of IFNs. Surprisingly, IFNs had little effect on the upregulation of PD-L1 on PANC-1, and the reasons for this are unknown. However, in the previous chapter I described how PD-L1 expression was predominantly found on insulin-producing β-cells with little to no expression in the exocrine cells. Western blotting demonstrated that PD-L1 is expressed on PANC-1 cells and is indeed present but is not as responsive to IFN as endocrine cells.

The discovery of PD-L1 on the surface of EVs from EndoC-βH1 cells is of interest because these EVs may play a role in attenuating the immune assault. Within tumour environments, exosomal PD-L1 is well positioned to mediate PD-1 crosslinking and immunosuppression because the exosome may not only express multiple PD-L1 molecules but also other proteins involved in T cell signalling such as MHC molecules. However, in the case of T1D, this mechanism may present both positive and negative effects because the HLA class I molecules have also been found on the surface of exosomes in some cancer studies (Daassi et al., 2020). While β-cell exosomal PD-L1 expression may suppress the immune attack, HLA Class molecules may draw the CD8+ T cells in (Figure 3-19). Autoreactive CD8 T cells are activated through interaction with peptides presented by HLA class I and can mediate β-cell death in a contact-dependent manner (Trivedi et al., 2016, DiLorenzo et al., 1998, Richardson et al., 2016). However, irrespective of HLA Class I molecules, the release of PD-L1 in exosomes potentially enhances the defensive mechanisms towards the immune cells. Researchers have typically focused on β-cells and

immune cells, but there is a complex system of vesicles constantly being transmitted between the two groups. Exosomes may well act as messengers that can direct immune cell behaviour.

There are additional implications for the role of EVs in T1D, particularly around insulin secretion, miRNA transport and virally-triggered T1D. Coxsackievirus B has previously been shown to escape from infected cells by ejected mitophagosomes and exosomes (Sin et al., 2017, Robinson et al., 2014) and this may occur in the pancreas to facilitate persistent infection.

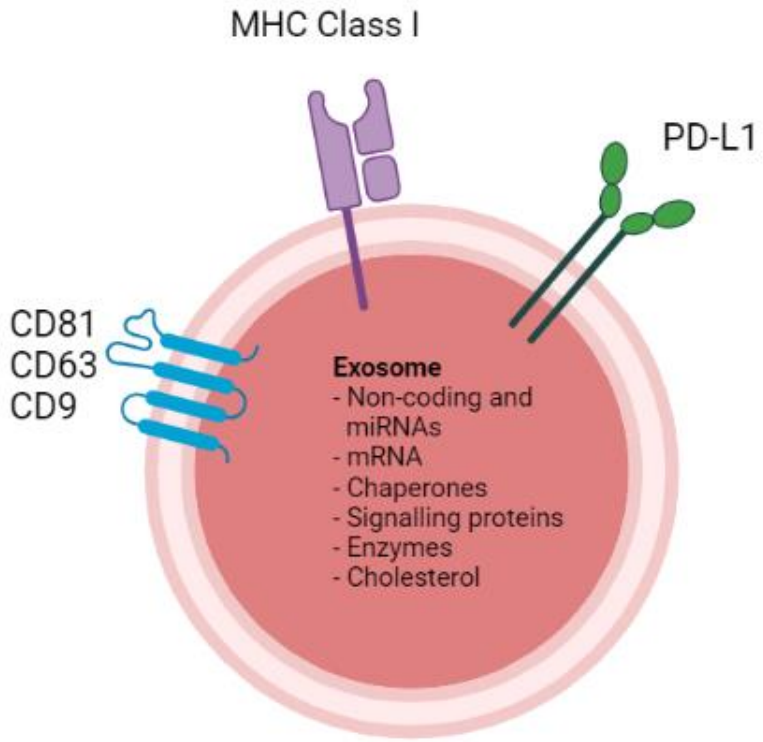


Figure 3-19 Cells expressing PD-L1 can produce extracellular PD-L1. PD-L1 inside the cell can be included on exosomes that are produced by the endocytic pathway and released by fusion of a multivesicular body with the cell membrane. Microvesicles containing PD-L1 can be produced by budding from the cell surface.

3.4.4 Clinical implications

Exosomes and EVs have the potential to become biomarkers of T1D progression. Studies have shown that exosomal PD-L1 can act as a marker of poor outcome after surgery or chemotherapy and radiation from patients with gastric cancer and head and neck squamous cell carcinoma (Fan et al., 2019, Theodoraki et al., 2018). If distinct markers of β-cells can be characterised, EVs could be collected in the blood or urine of people with T1D to provide insight into the health of the islets. In the previous chapter, I demonstrated that PD-L1 expression positively correlated with the level of immune infiltration of islets. Exosomes known to originate from endocrine cells expressing high levels of PD-L1 could indicate active immune activity against the islets. Low levels of expression could also indicate periods of remission. IBM^{TM} are currently developing new methods (the nanoscale deterministic lateral displacement (nanoDLD) chip) to support fast, reproducible, and automatable EV-isolation for early-stage disease diagnosis and treatment monitoring (Smith et al., 2018).

In this chapter, I have established a methodology to isolate EVs from pancreatic cells, helped confirm that 1.1B4 cells are rodent in origin, and confirmed that PD-L1 can be released in extracellular vesicles from EndoC-βH1 cells. One could speculate that this extracellular form of PD-L1 is also important for delaying the immune attack on β-cells in individuals with T1D. Interestingly, while EndoC-βH1 cells abundantly expressed PD-L1 upon stimulation by IFN-γ, the expression of PD-L1 in PANC-1 cells was much lower (Figure 3-18). In **Chapter two**, there was little (if any) expression of PD-L1 in the exocrine tissue, but PANC-1 cells are a human pancreatic cancer cell line isolated from a pancreatic carcinoma of ductal origin. This is interesting considering that many tumour cell types upregulate PD-L1 and are responsive to IFNs. Due to the

limited number of available human pancreatic cell lines, PANC-1 cells are an important resource for research in diabetes. However, pancreatic cancer (particularly pancreatic ductal adenocarcinoma) is a very complex disease and we have yet to fully understand how it alters the pancreatic cell. The following chapter delves more deeply into pancreatic ductal adenocarcinoma and its link with diabetes.

Chapter 4

4 Pancreatic ductal adenocarcinoma (PDAC) and diabetes

4.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most prevalent neoplastic disease of the pancreas accounting for more than 90% of all pancreatic malignancies (Hruban et al., 2007). PDAC is also the most lethal form of pancreatic cancer with survival typically ranging from 4 to 6 months following diagnosis, and the 5-year survival rate for all patients is 3-5% (Siegel et al., 2016, Bengtsson et al., 2020). Poor patient outcomes are a result of delayed diagnosis and an absence of effective therapeutic treatments. Identifying early markers of PDAC and understanding the cellular mechanisms driving tumour escape from both the immune system and immunotherapy are of great interest to researchers in the field (Orth et al., 2019).

Interestingly, there is a well-documented association between PDAC and diabetes, a form of diabetes sometimes referred to as type 3c diabetes mellitus (T3cDM) because it does not confidently satisfy the qualifying characteristics of either T1D or T2D. Pancreatogenic diabetes is classified by the American Diabetes Association and by the World Health Organization as T3cDM and refers to diabetes resulting from impairment in pancreatic endocrine function related to pancreatic exocrine damage from acute, relapsing and chronic pancreatitis (of any etiology), cystic fibrosis, hemochromatosis, pancreatic cancer, and pancreatectomy. It also refers to rare causes such as neonatal diabetes resulting from pancreatic agenesis (American Diabetes Association, 2012). Indeed, for many years there has been a debate as to the true physiological identity of this diabetes (i.e. similarity to T2D), the cellular drivers behind its manifestation and the potential it holds as an early warning sign of PDAC.

The association between PDAC development and diabetes has been observed over several decades. One study reports that about 45% to 65% of patients with PDAC have diabetes at diagnosis (Sharma and Chari, 2018). This contrasts with far fewer rates of diabetes developing with other non-pancreatic cancers and age-matched controls (Pannala et al., 2008, Aggarwal et al., 2013). On the other hand, epidemiological studies have also shown an increased risk of PDAC development in the T2D and T1D diabetic populations (Wolpin et al., 2013). Long-standing T2D is a (modest) risk factor for pancreatic cancer, indicating that duration of diabetes is important with five+ years considered to carry slightly higher risks than under five years (Wolpin et al., 2013) . These facts illustrate the “dual causality” relationship between diabetes and PDAC, with long-standing T2D being a risk factor for the development of PDAC and conversely PDAC causing diabetes. However there continues to be much debate about this dual causality in the literature, as some believe that all non-T1D forms of diabetes are T2D (Andersen et al., 2017).

PanCAN’s Early Detection Initiative is a group of researchers looking for clinical criteria that would potentially indicate a higher likelihood of undiagnosed PDAC. They are currently studying whether diagnostic imaging at the time of new-onset diabetes leads to earlier detection of PDAC. The group advises that those with new onset diabetes after the age of 50 have nearly a 1% chance of being diagnosed with PDAC within three years, and in these cases, the pancreatic tumour is believed to cause the diabetes which is a ‘symptom of cancer’. Around 25% of PDAC patients develop diabetes between 6 and 24 months prior to cancer diagnosis (Aggarwal et al., 2012a) and a recent study showed hyperglycemia precedes PDAC diagnosis by ~36 months, providing a possible window of opportunity for early detection of cancer (Maitra et al., 2018). By

some means, the pancreatic cancer is causing the process of hyperglycemia but through potentially dissimilar mechanisms to T2D (https://www.pancan.org/news/6-things-need-know-diabetes-pancreatic-cancer/ accessed 15/7/2021).

One important observation that supports the view that tumour growth can trigger diabetes is the length of time between diagnosis of diabetes and PDAC, which is often less than 24 months. Perhaps more compelling are the data showing that new-onset diabetes associated with PDAC may resolve following tumour resection, provided there are sufficient islets left in the residual pancreatic tissue. In a study of 104 patients who underwent PDAC resection, of whom 41 had diabetes at the time of surgery, it was found that 57% of the patients with new-onset diabetes had resolution of their diabetes postoperatively, whereas all of the patients with long-standing diabetes had a persistence of diabetes after pancreatic resection (Pannala et al., 2008, Andersen et al., 2017).

Despite its name, the originating cell of PDAC is contested, but a recent study using mouse models demonstrated that PDAC can originate from both ductal and acinar cells, albeit with distinct modes of progression (Ferreira et al., 2017). Described as an aggressive cancer, PDAC rapidly spreads to the lymphatic system and throughout the organs, which contributes to its poor prognosis. Additional risk factors for PDAC include advanced age, smoking, and chronic pancreatitis (Everhart and Wright 1995; Fuchs et al. 1996; Gapstur et al. 2000; Michaud et al. 2001; Berrington de Gonzalez et al. 2003; Stolzenberg-Solomon et al. 2005). Associations with genomic and somatic mutations are the most important factors driving aggressive phentoypes and the most common gene mutations associated with PDAC include *KRAS2*, *P16*, *TP53*, and *SMAD4* (Ahmed et al., 2017, Hansel et al., 2003). Additionally, chronic pancreatitis and

genes that predispose an individual to pancreatitis are associated with increased occurrence of PDAC. For example, patients with germline mutations in the gene PRSS1 have a 53-fold increased risk of developing PDAC (Lowenfels et al. 1997). This gene encodes an enzyme, cationic trypsinogen, whose hyperactivity causes a prolonged intrapancreatic effect of trypsin. This leads to destruction of the pancreatic tissue promoting diabetes, recurrent pancreatitis and PDAC.

In contrast to many other cancers, PDAC does not respond well to immunotherapy. In fact, PDAC stands out amongst cancers for its incredibly poor responses to checkpoint inhibitor therapy (ex. anti-PD-1 and anti-PD-L1 therapy) and monoclonal antibody therapy (Fan et al., 2020). The mechanisms underlying PDAC resistance to immune check-point inhibitors remain elusive, and as such represent an area of great interest for researchers. To date, there has been no conclusive evidence to suggest T3cDM influences immunotherapy outcomes, but in 2017 a group suggested that T3cDM could render patients more sensitive to such therapies when compared to PDAC patients without diabetes. The suggested reason behind this was the presence of an 'immunodeficient' phenotype from upregulation of PD-1 and CTLA-4 on PDAC tumour cells (Yan et al., 2017). Higher levels of PD-1 and CTLA-4 could potentially be suppressing the immune system from clearing tumour cells, and thus anti-PD-1 therapy may be a more effective form of treatment for PDAC patients with diabetes.

To date, the majority of studies that have assessed the genetic profiles of human PDAC have been conducted using whole tumour masses. These tumour masses contain the malignant pancreatic cells, but also immune cell infiltrates (e.g. T cells, B cell, macrophages etc.) and fibroblasts. These make up the

tumour microenvironment supporting tumour progression (Peng et al., 2019). Through use of this bulk tissue, researchers have gained a better understanding of the transcriptional networks and molecular mechanisms of PDAC. RNA expression profiles have enabled the categorization of PDAC into four subtypes; squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX) (Table 4-1). Importantly, these four subtypes of PDAC were found to be associated with varying clinical outcomes (Bailey et al., 2016).

The identification of four subtypes introduces new questions as to how, or if, mechanisms causing diabetes may influence the propensity for tumors to fall into one or two particular subtypes, by way of gene categorization. The methods undertaken to differentiate these subtypes did not exclude samples by clinical features such as diabetes. This could be important because the gene signatures associated with a progression of diabetes and endocrine dysfunction may be interpreted as reflecting a tumour phenotype, rather than the pathology of diabetes. Indeed, Yan et al. 2017 believed that the immunogenic class of PDAC was derived from the subgroup of PDAC patients with diabetes. However, their study did not include curation steps to remove non-PDAC pancreatic cancers from their sample groups, plus any contributing pathological conditions, such as chronic pancreatitis, that can also be associated with the development of diabetes.

Subtype	Gene of interest	Characteristics	Median survival (months)
Squamous	Upregulation of: • TP63, TP63ΔN Downregulation of genes that govern pancreatic endodermal cell-fate determination • PDX1, MNX1, GATA6, HNF1B Enriched for activated α6β1 and α6β4 integrin signalling, and activated EGF signalling	Gene networks involved in inflammation, hypoxia response, metabolic reprogramming, TGF-β signalling, MYC pathway activation and autophagy	30.00
Pancreatic progenitor	Upregulation of: • PDX1, MNX1, HNF4G, HNF4A, HNF1B, HNF1A, FOXA2, FOXA3 and HES1 Apomucins MUC5AC and MUC1 (not MUC2 or6) are preferentially co-expressed Downregulation of: • TGFBR2	Defined by transcriptional networks and transcriptional factors. These transcription factors are pivotal for pancreatic endoderm cell-fate determination towards a pancreatic lineage and are linked to maturity onset diabetes of the young (MODY). Gene programmes regulating fatty acid oxidation, steroid hormone biosynthesis, drug metabolism and O-linked glycosylation of mucins	25.6
Immunogenic	Upregulation of: • CTLA4 and PD-1 acquired tumour immune suppression pathways Associated immune gene programmes included B cell signalling pathways, antigen presentation, CD4+ T cell, CD8+ T cell and Toll-like receptor signalling pathways	The immunogenic class shares many of the characteristics of the pancreatic progenitor class but is associated with evidence of a significant immune infiltrate. Associated immune gene programmes included B cell signalling pathways, antigen presentation, CD4+ T cell, CD8+ T cell and Toll-like receptor signalling pathways	13.3
Aberrantly differentiated endocrine exocrine (ADEX)	Upregulation of: • transcription factors NR5A2, MIST1 (also known as BHLHA15A) and RBPJL and their downstream targets that are important in acinar cell differentiation and pancreatitis/regeneration	The ADEX class is defined by transcriptional networks that are important in later stages of pancreatic development and differentiation and is a	23.7

	• genes associated with endocrine differentiation and MODY (including INS, NEUROD1, NKX2-2 and MAFA. • transcription factors NR5A2, MIST1, RBPJL and their downstream targets that are important in acinar cell differentiation and pancreatitis/regeneration • cell lines expressed multiple genes associated with terminally differentiated pancreatic tissues, including AMY2B, PRSS1, PRSS3, CEL and INS.	subclass of pancreatic progenitor tumours. Transcriptional networks that characterize both exocrine and endocrine lineages at later stages are upregulated, rather than one or the other as is the case in normal pancreas development.	

Table 4-1. Brief summary of the four subtypes of pancreatic cancer as defined by Bailey et al. (2016).

A separate group took an alternative approach to Bailey et al. 2016 and focussed on the tumour cells, conducting single-cell transcriptomics to understand the heterogeneity of malignant cells that make up the tumour bulk, and discover the factors regulating PDAC progression (Peng et al., 2019). As expected, the tumour mass was found to be highly heterogeneous with diverse malignant and stromal cell types. Two ductal cell types with different transcriptomic profiles were identified with different clinical pathological features. Additionally, as well as previously implicated genes in PDAC (ex. *PI3K*, *Hedgehog*, *Wnt* and Notch pathways) they identified further novel genes such as *EGLN3*, *MMP9* and *PLAU*. They also identified transcription factors *FOS*, *KLF5* and *HMGA1*, the latter was associated with tumour progression, advanced tumour grade and decreased survival.

In addition to known cell type markers, such as *KRT19* for ductal cells and *PRSS1* for acinar cells (Segerstolpe, A. et al, 2016) Peng et al. (2019) also identified additional markers such as *TFF2* and *CELA3A* for ductal and acinar

cells respectively. *CHGB* was identified as a marker of the PDAC landscape for endocrine cells and is a gene that encodes a tyrosine-sulphated secretory protein abundant in peptidergic endocrine cells and neurons. As reported elsewhere, markers of poor prognosis in PDAC included *KRT19* (Yao, H. et al, 2016) and *CEACAM1/5/6* (Gebauer, F. et al, 2014).

Clinically, distinguishing T2D from PDAC-associated diabetes would be invaluable for early detection of PDAC, given the short survival rate. Anderson et al. 2017 previously described distinguishing features of PDAC-associated diabetes development, including the criterion that exocrine insufficiency predates the development of endocrine insufficiency. Previously, the diagnosis of PDAC-associated diabetes required the presence of exocrine insufficiency, evidence of pathological pancreatic imaging, and the absence of T1D-associated autoimmune markers (Table 4-2) (Andersen DK. 2012; 2016). The caveat to this approach is that it has relied on studies where PDAC-associated diabetes was also highly associated with a history of recurrent or chronic pancreatitis (Table 4-3). Previous reports have suggested that up to 76% of PDAC-associated diabetes can be related to chronic pancreatitis (Cui and Andersen, 2011), but further studies are needed to confirm this number.

The clinical observation of exocrine insufficiency occurring in conjunction with pancreatitis prior to the diagnosis of PDAC is compelling and it has been suggested that exocrine dysfunction could promote the release of growth factors, cytokines and reactive oxygen species that promote tumorigenesis. However, chronic pancreatitis is often associated with germline mutations that are also risk factors for PDAC, such as *PRSS1*, and it is also a significant risk factor for T3cDM. What has often been overlooked in the literature, is the difficulty in determining if PDAC-associated diabetes is more associated with

tumour growth or with chronic pancreatitis. Despite an extensive literature search, I have not seen published research that investigates PDAC-associated diabetes under conditions where chronic pancreatitis has been removed as a contributing factor. However, this may be due to limited numbers of PDAC samples. For this reason, I decided to look at the genetic characteristics of patients with PDAC-associated diabetes but no history of pancreatitis.

Major criteria
Presence of exocrine pancreatic insufficiency (according to monoclonal faecal elastase 1 or direct function tests).
Pathological pancreatic imaging (by endoscopic ultrasound, MRI, or computed tomography).
Absence of T1DM-associated autoantibodies
Minor criteria
Impaired β-cell function (e.g., as measured by HOMA-B, C-peptide/glucose ratio).
No excessive insulin resistance (e.g., as measured by HOMA of insulin resistance).
Impaired incretin (e.g., GIP) or PP secretion.
Low serum levels of lipid soluble vitamins (A, D, E, or K).

Table 4-2 Diagnostic criteria for T3cDM (adapted from Andersen et al. (2017).

Parameter	T1DM	T2DM	T3cDM
Ketoacidosis	Common	Rare	Rare
Hypoglycemia	Common	Rare	Common
Peripheral insulin sensitivity	Normal or decreased	Decreased	Normal or increased
Hepatic insulin sensitivity	Normal or decreased	Decreased	Normal or decreased
Insulin levels	Low or absent	High or "normal"	"Normal" or low
Glucagon levels	Normal or high	Normal or high	"Normal" or low
PP levels	Normal or low (late)	Normal or high	Low or absent
GIP levels	Normal or low	Variable	Low
GLP-1 levels	Normal	Variable	Variable
Typical age of onset	Childhood or adolescence	Adulthood	Any
Typical etiology	Autoimmune	Obesity, age	CP, cystic fibrosis, postoperative

Table 4-3 Clinical and laboratory findings in types of diabetes; T1D, T12, and T3cDM (adapted from Andersen et al. (2017).

The mechanisms of interaction between pancreatic β-cells, the infiltrating immune cells and other pancreatic cell types during the development of diabetes that were explored within the previous chapters of this thesis have here been extended to the juxtaposition with pancreatic cancer.

The broad aim of this Chapter was to determine whether patients with PDAC and diabetes, and no history of chronic pancreatitis, predominantly fall into a class of PDAC due to gene expression of markers (Table 4-1) more associated with the pathology of the diabetes rather than PDAC.

To do this, I curated data from The Cancer Genome Atlas (TCGA) to create two samples groups: PDAC patients with no history of diabetes, and PDAC patients with diabetes plus no history of pancreatitis. I then analysed the gene expression data for each cohort. The TCGA was a large-scale collaboration between the National Cancer Institute and the National Human Genome Research Institute to comprehensively characterize the molecular events in primary cancers. This effort, which ended in 2017, generated publicly available comprehensive, multidimensional maps of genomic changes in >11,000 tumor samples across 33 types of human tumors.

4.2 Methods

4.2.1 Patient Data Collection

Patient data was retrieved from The Cancer Genome Atlas (TCGA). This provides comprehensive, multidimensional maps of genomic changes in >11,000 tumor samples. Level 3 RSEM normalized Illumina HiSeq RNA expression data. RSEM is an acronym for RNA-seq by Expectation Maximization (Li & Dewey, 2011). The TCGA/PanCancer Atlas pancreatic ductal adenocarcinoma (PDAC) cohort was downloaded from the Broad Genome Data Analysis Centers Firehose server

(https://gdac.broadinstitute.org/). The gene level Firehose dataset was used for all genes. Written informed consent was obtained from the patients (or their families) by the TCGA project. Normalized expression data was imported into Microsoft Excel and patient cohorts were manually curated to remove those with a history of pancreatitis, or whose history was 'unknown'.

Firehose provides access to standardized higher-level data for each individual TCGA cohort as of the final build of 2016_01_28 on their server. For the purposes of this chapter, key files include mRNA-seq that have been analyzed and normalized using a standardized pipeline that allows comparison within and between TCGA cohorts. The files were freely downloaded from https://gdac.broadinstitute.org/. These files represent quantitative RNA-seq read value for each gene for each TCGA sample. Files downloaded from the archives were designated as RSEM-normalized. The use of mRNA expression data that has undergone RSEM normalization via a uniform pipeline allows comparison of samples within and between TCGA cohorts.

The clinical archive includes basic patient data like age, gender and race, information on clinical outcomes and specialized data related to treatment modality, tumor pathology characteristic, smoking status, etc.

4.2.2 Tissue Analysis

Images of tissue samples for all TCGA samples are freely available for analysis using the Cancer Digital Slide Archive (http://cancer.digitalslidearchive.net). Fixation methods were non-standardized and collection occurred over multiple laboratory sites.

4.2.3 Statistics

Boxplot comparison of gene expression was performed using GraphPad Prism v7.0 (Graphpad Software, Inc., San Diego, California, USA). For the boxplots,

centre lines show the medians, box limits indicate the 25th and 75th percentiles, and whiskers extend from the minimum to maximum values. Statistical significance ($p<0.05$) was calculated using a one-tailed non-parametric Mann–Whitney U test.

The limited availability of normal control tissues in the PDAC TCGA cohort meant that any analysis with those samples was underpowered and lack of significance is inconclusive due to insufficient sample numbers. Therefore, I proceeded with my analysis comparing the diabetic PDAC patient cohort with the non-diabetic PDAC patient cohort. In total I had 22 PDAC with associated diabetes information, and 84 PDAC samples with no associated diabetes. As stated above, the cohort with PDAC-associated diabetes was curated to remove all patients with a clinical description of 'history of pancreatitis' or 'unknown history of pancreatitis'. Additionally misclassified samples (i.e. non-PDAC cancers) were excluded from the study as indicated by Peran et al. (2018) to ensure integrity of PDAC sample population (Peran et al., 2018).

Statistical significance for correlation analysis was determined using Spearman's Rho analysis, with $p< 0.05$ as a cutoff.

Overall survival outcomes were compared in PDAC patients and dichotomized by presence or absence of diabetes. A log-rank statistical p-value was calculated for the Cox survival model using GraphPad Prism 8.4.3.

4.2.4 CIBERSORT

CIBERSORT is a free online software program that uses a 547 gene panel to deconvolute complex mixtures of cell types and distinguish 22 human hematopoietic cell phenotypes. This analysis estimates the relative levels of seven T-cell types, naïve and memory B cells, plasma cells, NK cells, and

myeloid subsets (Newman et al., 2015). The CIBERSORT algorithm generates a false discovery rate (FDR) value, providing a statistical measure of confidence for the estimation of each of the 22 immune cell types in each TCGA sample.

4.3 Results

4.3.1 PDAC donor samples show significant levels of fibrosis and collagen around the islets

The TCGA program occurred over a timeframe of 10-years, which for the quantity of data collected was a relatively short period of time. Being a large-scale study with samples collected over multiple locations, the acquisition of comprehensive clinical annotation was not always possible for every TCGA sample. Nevertheless, substantial clinical data are available and allow for many clinical variables to be assessed. Within the sets of data available for each patient, there are available tissue slides and pathology reports for the specimens collected.

I wished to investigate whether the islets in donors with diabetes and PDAC were morphologically different to tissue from donors without diabetes. Unfortunately, despite the online TCGA catalogue recording the availability of four samples of normal pancreatic tissue, upon closer inspection and verification with the pathology report, these samples showed signs of PDAC. They have therefore been misclassified. Therefore, I was unable to compare PDAC tissue sections to control pancreatic tissue sections but images from earlier chapters stained with DAB can provide an indication of a ‘healthy’ pancreas.

Sections of pancreatic tissue from donors were analyzed in clinical pathology labs across the U.S. so it is important to note that non-standardized fixation methods were used to preserve the tissue sections. The main purpose for

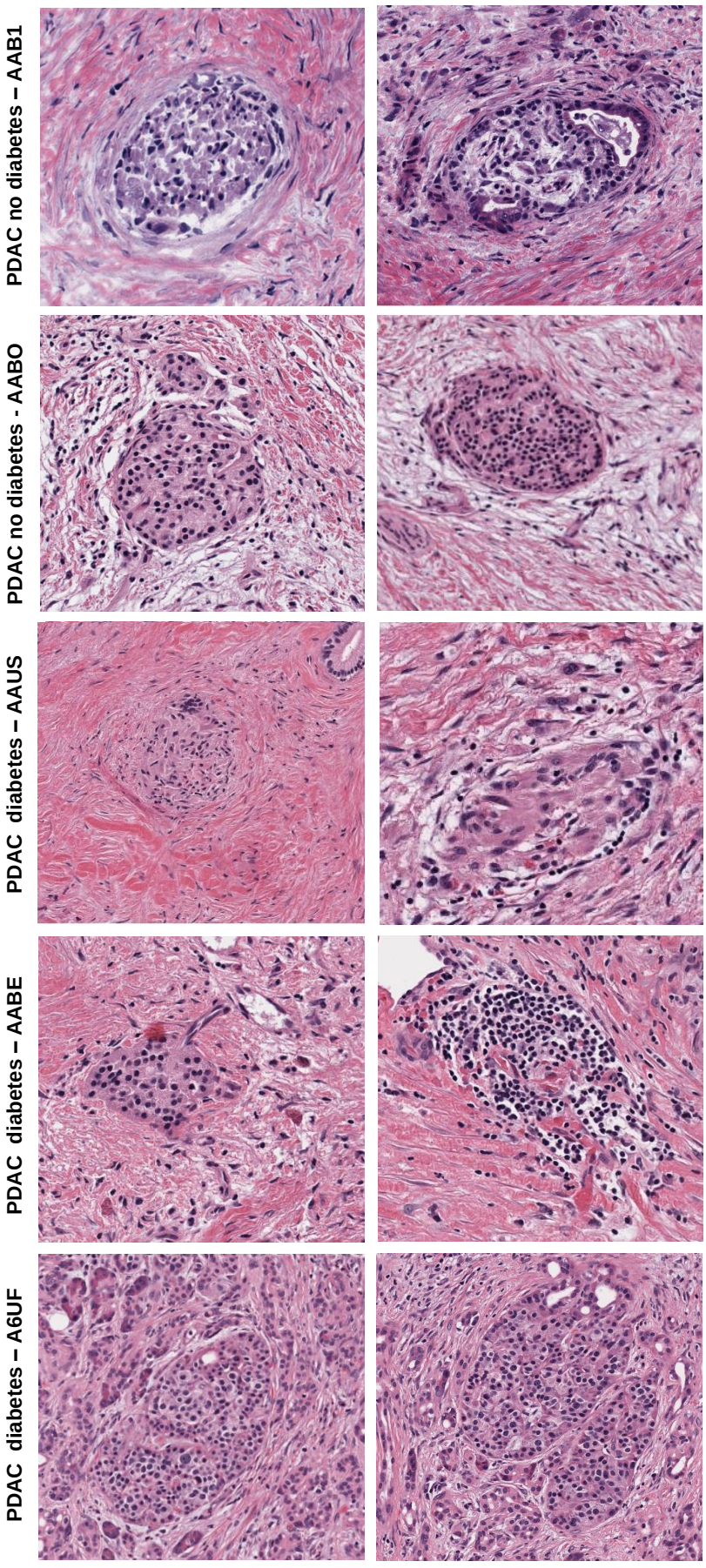
creating a database of tissue sections was to create a catalogue demonstrating the pathology of the tumours and to determine level of invasiveness, origins of growth and grade of cellular differentiation. PDAC is associated with marked fibrosis, type I collagen, and stomal myofibroblasts (Özdemir et al., 2014). The three dominant entities in the PDAC stroma are extracellular matrix (ECM), vasculature and cancer-associated fibroblasts (CAFs). The ECM has been observed to form a barrier to effective drug delivery to PDAC cells, the tumour vasculature is a complex system and PDAC CAFs participate in active cross-talk with cancer cells within the tumour microenvironment (Hosein et al., 2020). Interestingly, to the best of my knowledge, there is only one published study (Goess et al., 2021) that has examined islets within the tumour microenvironment across PDAC individuals, so there is limited knowledge on how the above three dominant entities affect islet function.

As these tissue sections have not been stained for insulin, I made informed decisions on identifying islets based on known islet characteristics such as an encompassing basement membrane. Within all tissue sections in Figure 4-1A, there are substantial levels of fibrosis. In the non-diabetic sample AAB1, the basement membrane appears to be unusually thick, and the internal architecture of the islet appears intact. When looking for islets across the two cohorts, I found the non-diabetic group had more easily identifiable islets, with distinct basement membranes.

The three representative tissue samples of PDAC with diabetes demonstrate different time points from diabetes diagnosis. AABE represents a diabetes diagnosis of 9 days prior to PDAC diagnosis, AAUS represents a diabetes diagnosis of 49 days and A6UF represents a diagnosis of 551 days. As summarized in the introduction, the development of diabetes shortly before the

diagnosis of PDAC (within 18 months) may be a result of cancer progression. The two recent onset cases of diabetes (AABE and AAUS) appear to have high levels of fibrosis in and around the islet and loss of islet definition (islet 2 of AAUS). The islets from the donor diagnosed with diabetes 551 days prior to PDAC diagnosis appeared to be more like the non-diabetic cohort.

A


PDAC no diabetes – AAB1
PDAC no diabetes - AABO
PDAC diabetes – AAUS
PDAC diabetes – AABE
PDAC diabetes – A6UF

B

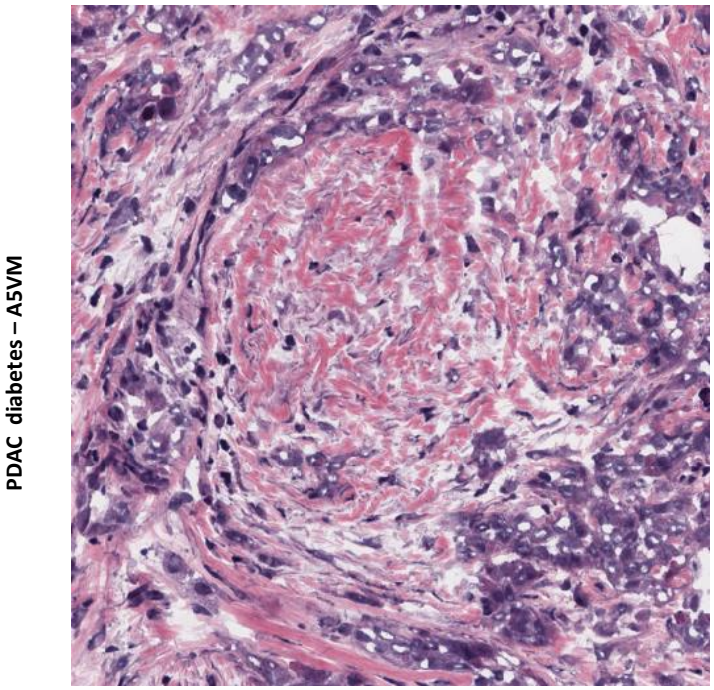


Figure 4-1 Islet pathology in PDAC. (A) Islets from individuals with PDAC are situated within high levels of fibrosis. Representative islets are shown from the cohort with no diabetes (AAB0 and AAB1) and the cohort with diabetes (AABE, AAUS, A6UF). AABE and AAUS represent recent-onset diabetes, while A6UF represents >1 year diabetes onset. All samples were taken from PDAC tumors graded stage IIB and located at the head of the pancreas. (B) Pancreatic tissue from an individual with PDAC and diabetes shows high levels of fibrosis and alteration of the exocrine tissue. Tissue images were retrieved from the TCGA database (accessed from https://portal.gdc.cancer.gov/).

4.3.2 Insulin, Glucagon and Somatostatin gene expression is lower in PDAC patients with diabetes

There are five major endocrine cells of the pancreas and these are found within the islets of Langerhans. *INS, GCG and SST* encode the islet-derived hormones insulin, glucagon, and somatostatin respectively, and all three genes were significantly reduced in patients with PDAC-associated diabetes when compared to PDAC donors without diabetes (Figure 4-2). *GCK* plays an important role in recognising how high the blood glucose is in the body and encodes the glucose sensor glucokinase. *GCK* was not found to significantly differ between the cohorts.

Correlation analysis confirmed that *INS* expression positively correlated with *GCG and SST*. This would be expected if whole islets were being affected as opposed to individual cell-types. Interestingly, length of diabetes (i.e. number of days since diagnosis) did not correlate with gene expression and donors with long-duration diabetes did not show reduced expression of *INS*. Donors with similar histories of diabetes had varied levels of endocrine cell gene expression (Figure 4-2). However, this may be a result of varied levels of tissue damage across the sample area.

WSCD2 was previously found to display a positive correlation with insulin levels in individuals with T2D. Analysis of the expression profile of *WSCD2* in an islet expression array revealed significant down-regulation in diabetic/hyperglycaemic donors compared with non-diabetic/normoglycemic. Importantly, this gene is expressed in β-cells at much higher rates compared with α-cells or exocrine cells (Taneera et al., 2014). A correlation analysis confirmed positive correlation between *WSCD2* and *INS* ($r = 0.56$, $p<0.006$) and *WSCD2* and *GCG* ($r = 0.52$, $p<0.01$).

The endocrine marker *CHGB* described by Peng et al. (2019) in PDAC tumors was also examined and found to be expressed at similar levels across the two groups.

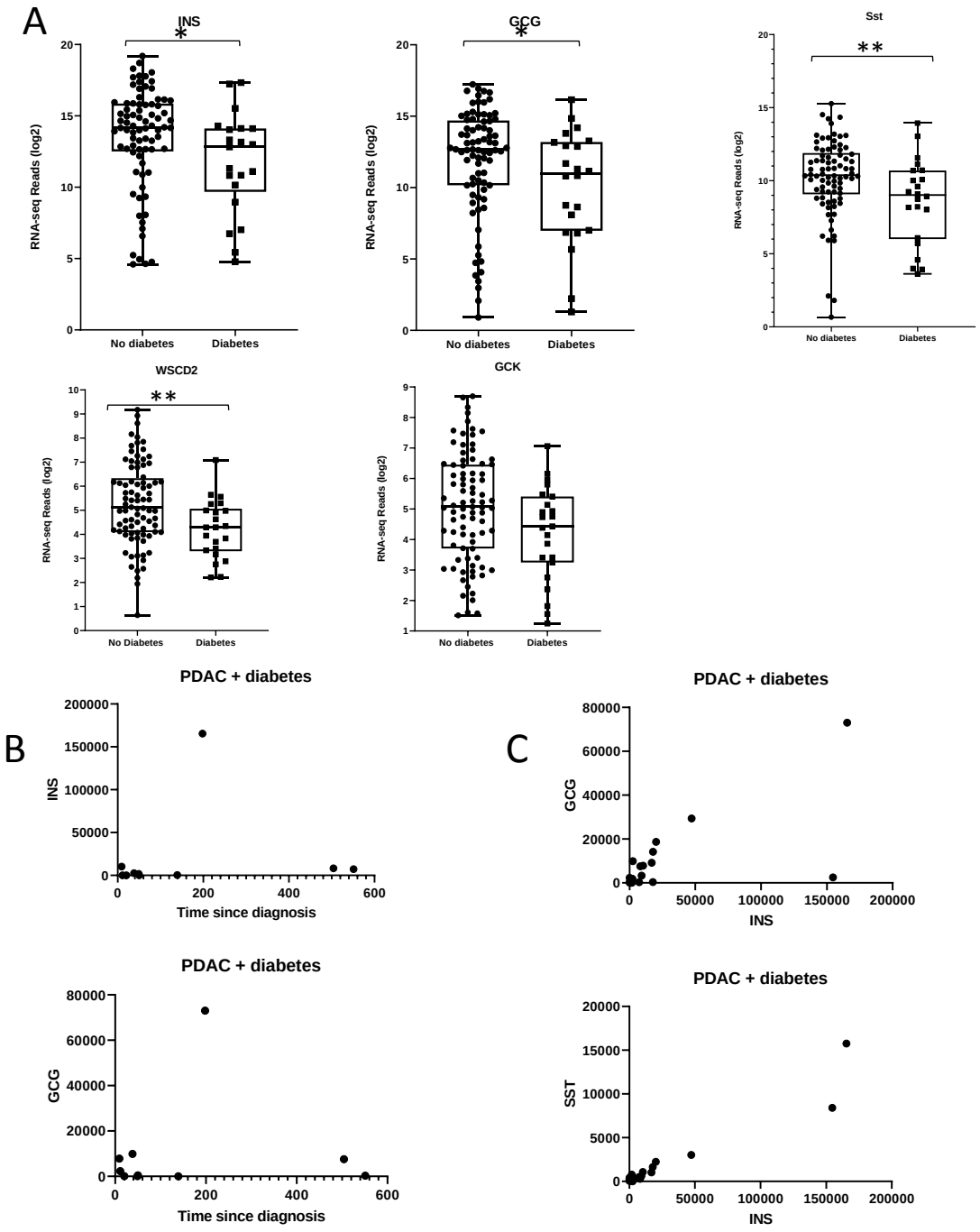


Figure 4-2 Gene expression analysis of endocrine markers across cohorts. (A) Endocrine markers *INS, GCG, SST,* and *WSCD2* were all reduced in the diabetic cohort (* p<0.05, ** p<0.01) (B) Correlation analysis between duration of diabetes and *INS* (r = 0.36); *GCG* (r = -0.06). (C) Correlation analysis between *GCG* and *INS* (r = 0.79; p<0.001), and *SST* and INS (r = 0.84; p<0.001) for the diabetic cohort.

4.3.3 Expression levels of genes associated with exocrine tissue are similar across both donor groups

The cells of the exocrine tissue produce digestive enzymes and chloride enriched fluid for transportation of enzymes. *CELA3A* and *AMY2A* are proteins expressed by exocrine cells and are involved in enzymatic digestion of proteins and carbohydates. Enzymes are transported through the pancreatic ductal system while the ductal epithelium regulates pH.

Gene markers for ductal cells *CELA3A*, *TFF2*, *CFTR*, *SCTR*, *DCDC2* were similar across both cohorts. The functions of these genes can be found in Table 4-4. *PRSS1*, a gene typically associated with acinar cells was found to be similar across both donor groups. Interestingly, no markers of fibrous tissue (collagen, elastin, fibronectin, fibrin) were found to significantly differ between the two cohorts.

Gene	Protein	Protein Function
CELA3A	Hymotrypsin-like elastase family member 3A	• serine-type endopeptidase activity
TFF2	Trefoil factor 2	• CXCR4 chemokine receptor binding • chemokine-mediated signalling pathway • maintenance of gastrointestinal epithelium • negative regulation of gastric acid secretion
CFTR	Cystic fibrosis transmembrane conductance regulator	• ATPase-coupled transmembrane transporter activity • ATP binding • intracellularly ATP-gated chloride channel activity • isomerase activity • Mediates the transport of chloride ions across the cell membrane.
SCTR	Secretin receptor	• G protein-coupled peptide receptor activity • peptide hormone binding • secretin receptor activity
DCDC2	Doublecortin domain-containing protein 2	• inhibition of canonical Wnt signalling pathway • kinesin binding

Table 4-4 Gene markers of ductal cells and their protein functions.

In contrast, *KRT19* gene expression by ductal epithelial cells was significantly lower in the PDAC-associated diabetes group (Figure 4-3). KRT19 is an intermediate type 1 keratin. Previous studies have found an association between upregulation of *KRT19* in PDAC tumour samples and lower survival rates in patients. (Bailey et al. 2019; Dugnani et al, 2018). However, after constructing a Kaplan-Meier curve to compare overall survival for patients with and without diabetes, no significant difference in clinical outcome between PDAC patients with diabetes and without diabetes (Figure 4-4).

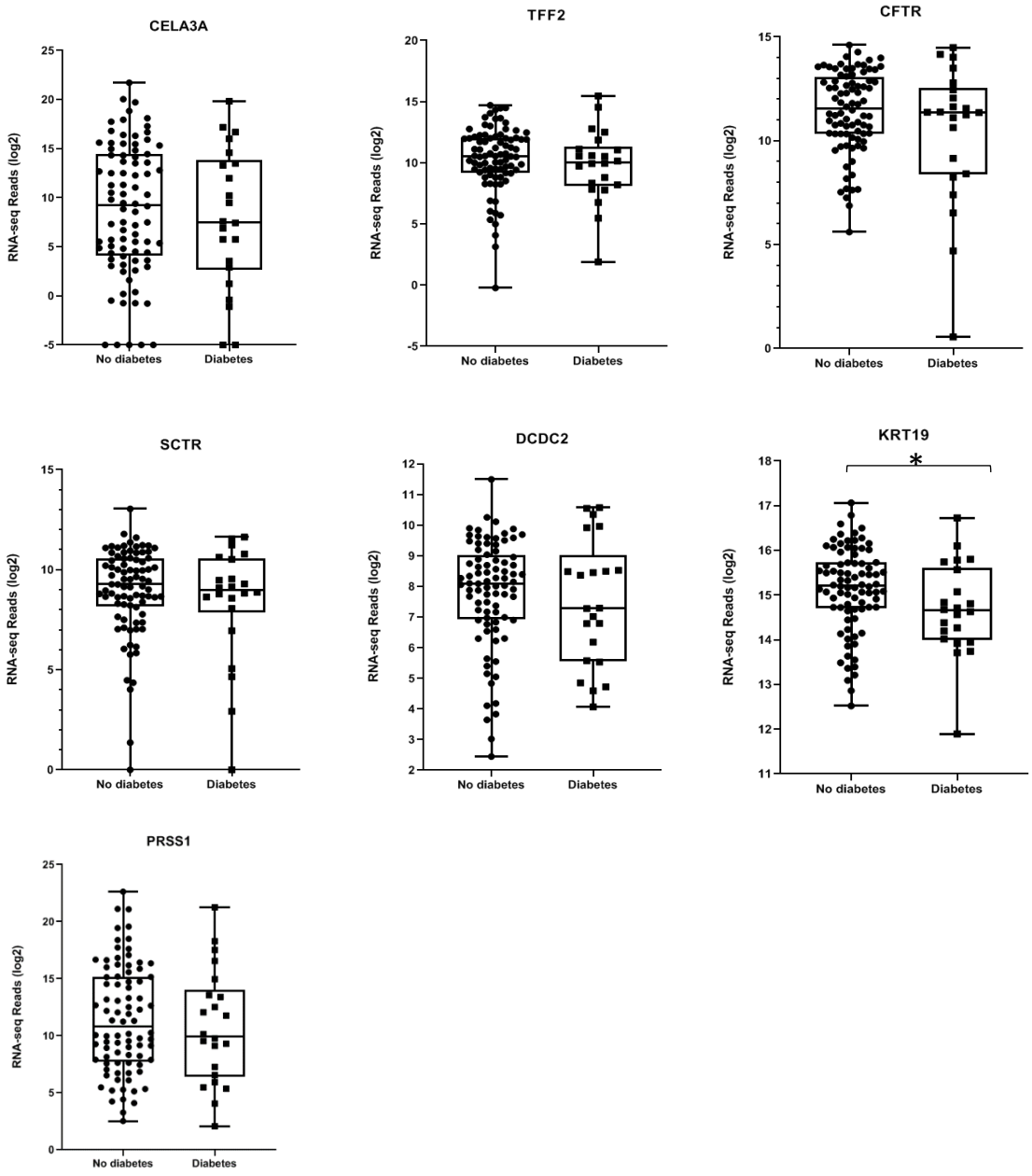


Figure 4-3 Gene expression analysis of exocrine markers across cohorts. Genes associated with the exocrine tissue had similar expression across cohorts except *KRT19*, which was significantly reduced in the diabetic cohort (* $p<0.05$).

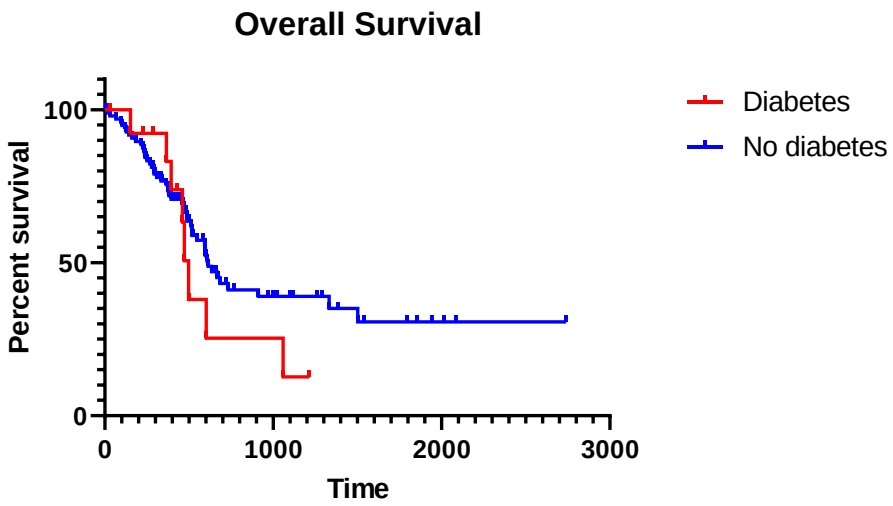


Figure 4-4 Comparison of clinical outcomes for PDAC with diabetes cohort versus no diabetes cohort. Clinical data was downloaded from the Firehose server and plotted as a Kaplan-Meier curve based on presence or absence of diabetes. Mean survival for the diabetes cohort = 498 days, no diabetes cohort = 614 days. Time = days, p = 0.3.

4.3.4 A diagnosis of diabetes is not associated with a subtype of PDAC

To determine if patients with diabetes were disproportionately developing a certain subtype of PDAC, gene lists from Table 4-1 were analyzed across both cohorts.

Firstly, I focused on genes typically associated with the squamous subtype: *TP53, MINX1, GATA6, ITGA6, HNF1B, and PDX1.* Analysis across both donor groups revealed no significant differences in any of these genes between cohorts (Figure 4-5). For this subtype, we would expect to see downregulation of genes that govern pancreatic endodermal cell-fate determination.

In other studies where PDAC bulk tumour was analysed for gene expression, reactivation of several embryonic genes was observed. One study found *PDX1* was upregulated in half of the PDAC samples and correlated with a poor prognosis (Koizumi et al. 2003). In my study, mean *PDX1* expression values were lower in the diabetes cohort, but this was not significant ($p = 0.09$).

I next focused on genes typically associated with the pancreatic progenitor subtype: *HNF4G, HNF4A, FOXA3, TGFBR2, HNF1B, FOXA2, and HES1.* From this set of genes, *HNF4G* and *FOXA3* were significantly reduced in the donor group with diabetes, which is contrary to what we would expect if these tumours were phenotypically more like the pancreatic progenitor subtype (Figure 4-6). *HNF4G* encodes for the protein hepatocyte nuclear factor 4-gamma, which functions as a transcription factor and operates with HNF4α to regulate islet transcription. Diseases associated with the HNF4 transcription factors include MODY and T2D (Odom et al., 2004). *FOXA3* encodes the protein forkhead box protein A3, which acts as a transcription factor and is also associated with MODY.

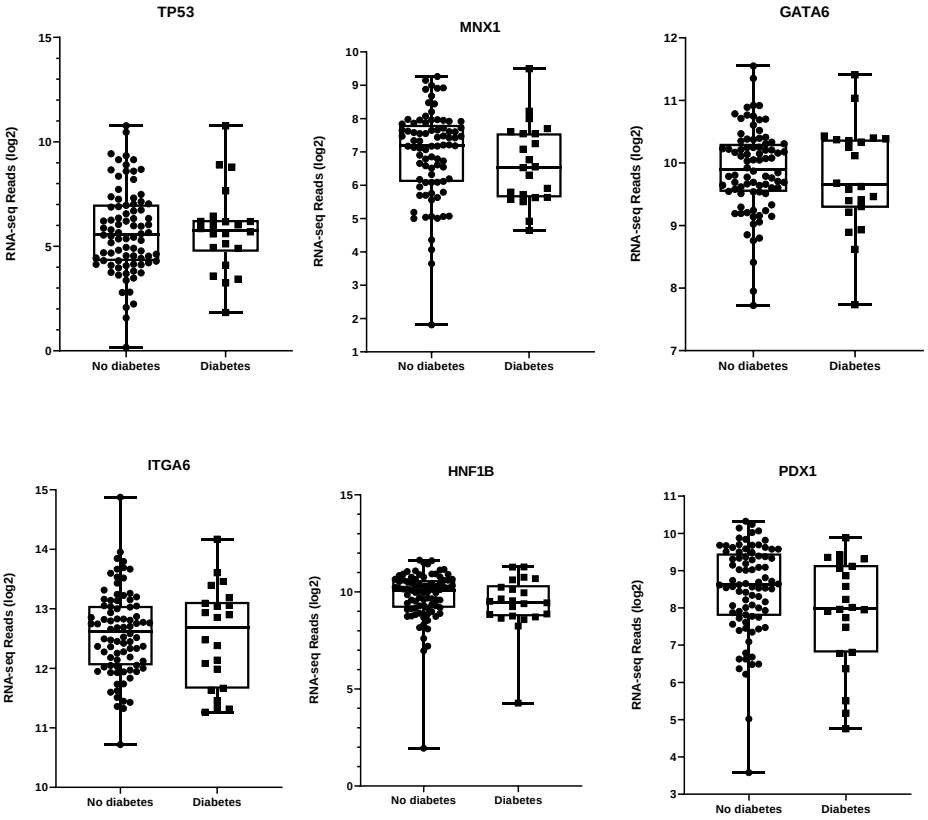


Figure 4-5 Gene expression analysis for genetic markers of the squamous subtype. Gene expression profiles for *TP53, MNXI*, *GATA6*, *ITGA6, HNF1B* and *PDX1* were similar across both PDAC cohorts.

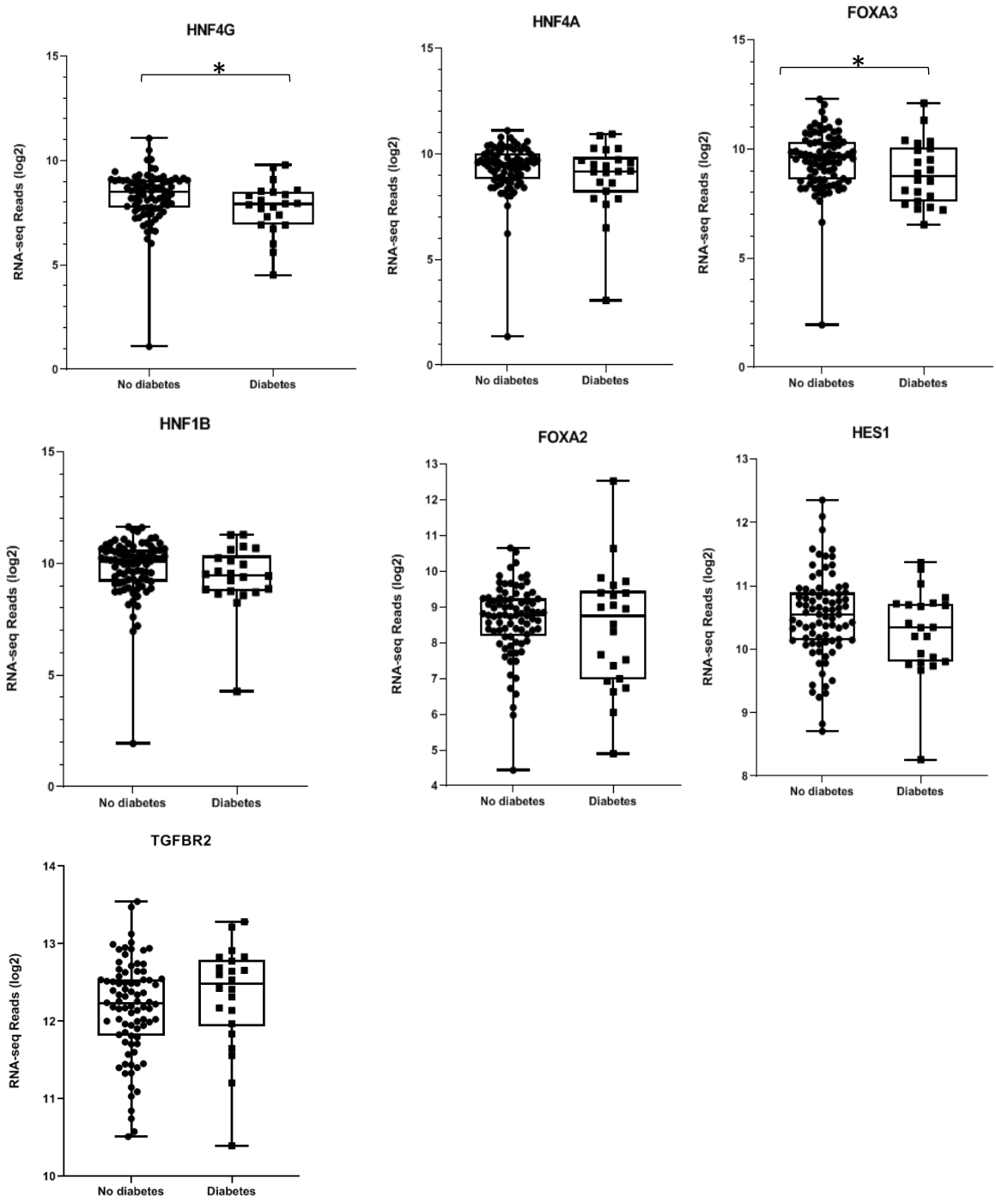


Figure 4-6 Gene expression analysis for genetic markers of the pancreatic progenitor subtype. Gene expression profiles for *HNF4G*, *HNF4A, FOXA3, HNF1B, FOXA2, HES1,* and *TGFBR2* were compared across both cohorts. *HNF4G* and *FOXA3* were significantly reduced in the diabetes cohort (*p<0.05).

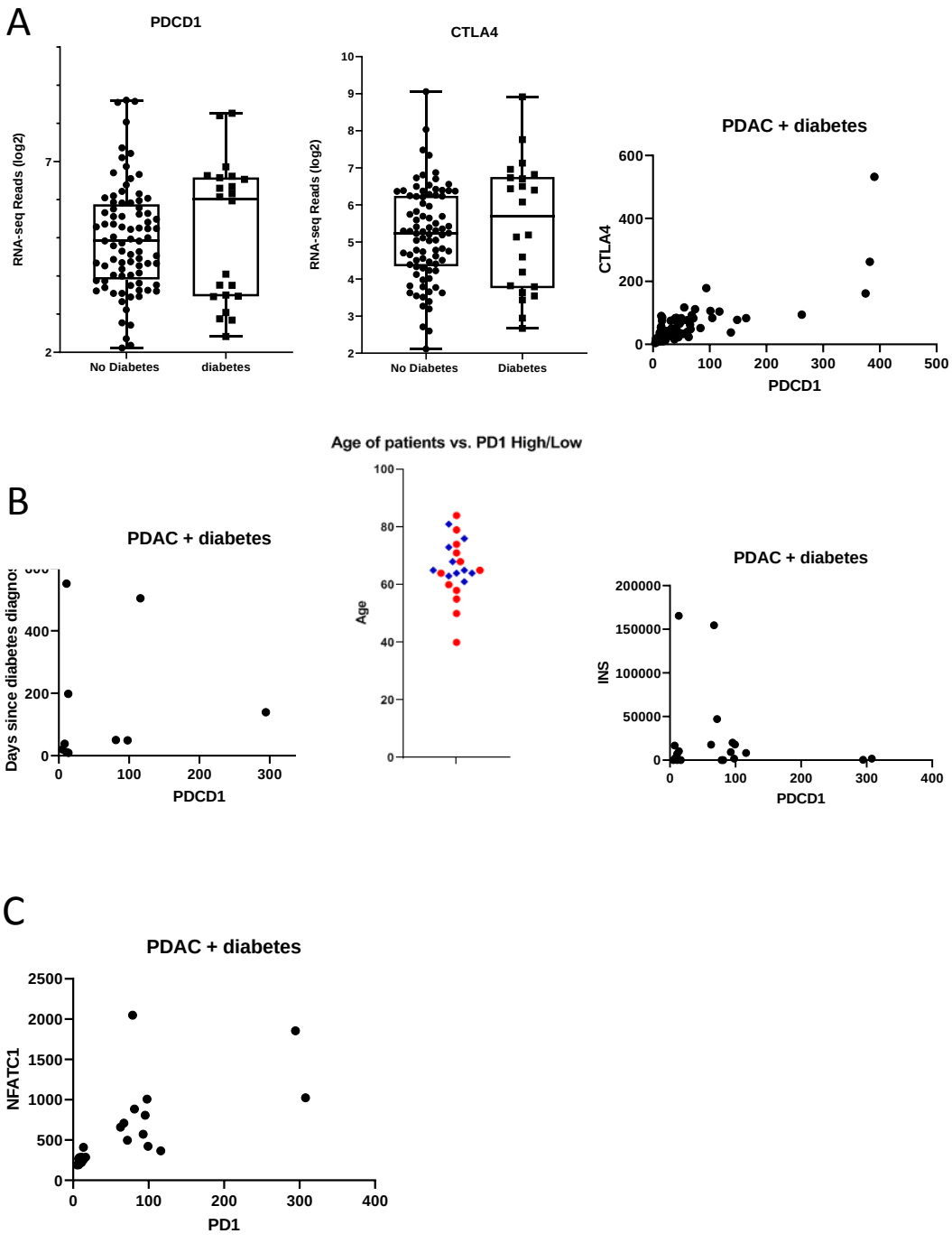

A
PDCD1
RNA-seq Reads (log2)
No Diabetes
diabetes
CTLA4
RNA-seq Reads (log2)
No Diabetes
Diabetes
PDAC + diabetes
CTLA4
PDCD1
B
PDAC + diabetes
Days since diabetes diagnos
PDCD1
Age of patients vs. PD1 High/Low
Age
PDAC + diabetes
INS
PDCD1
C
PDAC + diabetes
NFATC1
PD1

Figure 4-7 Gene expression analysis for genetic markers of immunogenic subtype. (A) *PDCD1* expression had a binary pattern in the diabetic group. Correlation analysis between *PDCD1* and *CTLA4* expression in the diabetic cohort was significant (r = 0.9, p<0.001) (B) No correlation was observed between *PDCD1* and duration of diabetes (r = 0.4) , similarly between *PDCD1* and *INS* in the diabetic cohort (r = 0.09). (C) Expression of *PDCD1* was significantly correlated with *NFATC1* in the diabetic cohort (r = 0.8, p<0.001).

To determine if the donor group with diabetes had gene expression profiles associated with the immunogenic subtype, I analyzed the gene expression levels of *PDCD1* and *CTLA4* (Figure 4-7A). Neither gene was significantly different across the donor groups, but interestingly, a clear binary distribution pattern can be seen in the analysis of the *PDCD1* group. There is very clearly a *PDCD1* high and a *PDCD1* low group. A correlation analysis between *PDCD1* and *CTLA4* was highly significant. These two genes encode immune checkpoint inhibitors (PD-1 and CTLA-4) which can typically be found on the same immune cells (as summarized in chapter two). Tumour infiltrating CD8+ T cells should express both genes and we expect that patient samples with high levels one would also express high levels of the other. I also conducted a correlation analysis between *PDCD1* expression, and a gene known to encode proteins upstream of the PD-1 receptor, namely the NFATC signaling pathway. *NFATC1* and *PDCD1* were found to be positively and significantly associated with *PDCD1* (Figure 4-7C).

To determine if duration of diabetes was associated with gene expression of *PDCD1*, a correlation analysis was run and showed that no significant association, but the *PDCD1* low group of patients were typically >60 years of age. Also, *PDCD1* expression in the diabetes cohort did not correlate with *INS* or *GCG* expression, indicating that islet function was not associated with the immunogenic subtype (Figure 4-7B).

To further confirm that *PDCD1* expression correlated with additional markers of immune cells, a correlation analysis was conducted between *PDCD1 and CD8, CD45, CD72* and *CD38.* All immune cell genes were found to be significantly correlated with expression of *PDCD1*, which further indicates the presence of immune infiltration (Figure 4-8). I also analyzed the correlation between *PDCD1*

and *PDL1*, the gene encoding the ligand binding partner to the PD-1 receptor on T cells. In the diabetes donor group, there was no significant correlation, indicating that even in the *PDCD1* high group, there was no corresponding increase in *PDL1* (Figure 4-8). The same analysis was run for the non-diabetic donor group, and a significant association was found ($p<0.05$). Upstream activators that lead to the transcription of PD-L1 include IL-6, IFN-α, and IFN-γ. When looking at the RNA expression, only *IL6* seems to be upregulated in the tumour microenvironment within the diabetes cohort ($p<0.05$). *PDL1* does not positively correlate with *IFNG*, but there is a very strong correlation between *PDL1* and *STAT1* levels ($p<0.001$), but not *STAT2*.

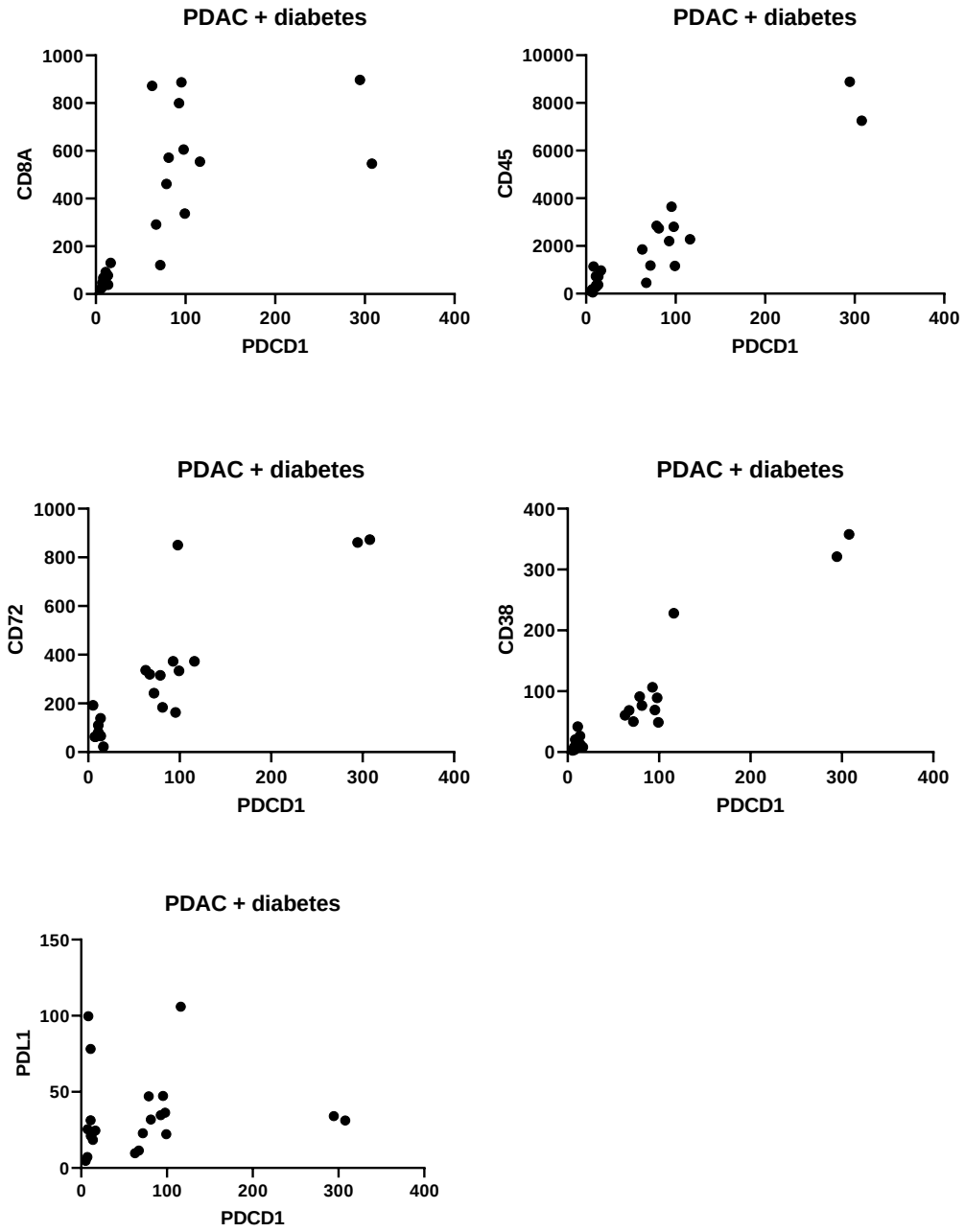


Figure 4-8 Correlation analysis between *PDCD1* and markers of immune cells and checkpoint inhibitors. Expression of *PDCD1* was found to significantly correlate with immune markers *CD8* (T cells) (r = 0.8), *CD45* (hematopoietic cells) (r = 0.87), *CD72* (B cells) (r = 0.8) and *CD38* (immune cell activation) (r = 09). *PDCD1* was not found to correlate with *PDL1* (immune checkpoint).

Finally, I analyzed the genes that characterize the ADEX subtype across both donor groups: *NR5A2, BHLHA15, NEUROD1, PRSS1, NKX2-2, MAFA,* INS, and *PAX4.* From this set of genes, *MAFA, PAX4* and *INS* were all significantly reduced in the diabetic group (Figure 4-9). *MAFA* is significantly reduced in the diabetic group and positively correlates with *INS* expression. MafA is a transcription factor and marker of mature β-cells. It binds to the insulin enhancer element RIPE3b/C1-A2 and activates insulin gene expression. *MAFA* has been shown to be downregulated in human studies of T2D (Guo et al., 2013).

In the study by Bailey et al. (2016), these genes were all upregulated in the ADEX group, so the presence of diabetes does not seem to increase the occurrence that these donors will have the ADEX subtype.

Correlation analysis between the genes *GCG* and *PAX4* in the diabetic donor group showed a significant association in expression (Figure 4-9). In gene knockout experiments with mice, *Pax4* has been shown to have opposing functions to *ARX*, a marker of mature alpha cells and represses ARX transcription (Collombat et al., 2003). *Pax4* knockout mice lack insulin and somatostatin positive cells but retain numerous glucagon-positive cells (Brink et al., 2001). Lower levels of P*AX4* in the diabetic group may reflect the lower levels of the *INS* gene. Transcription factors ARX, and MAFB in the diabetic group do not significantly correlate with *GCG* despite being markers of mature alpha cells. However, they are significantly correlated in the non-diabetic group ($p<0.001$).

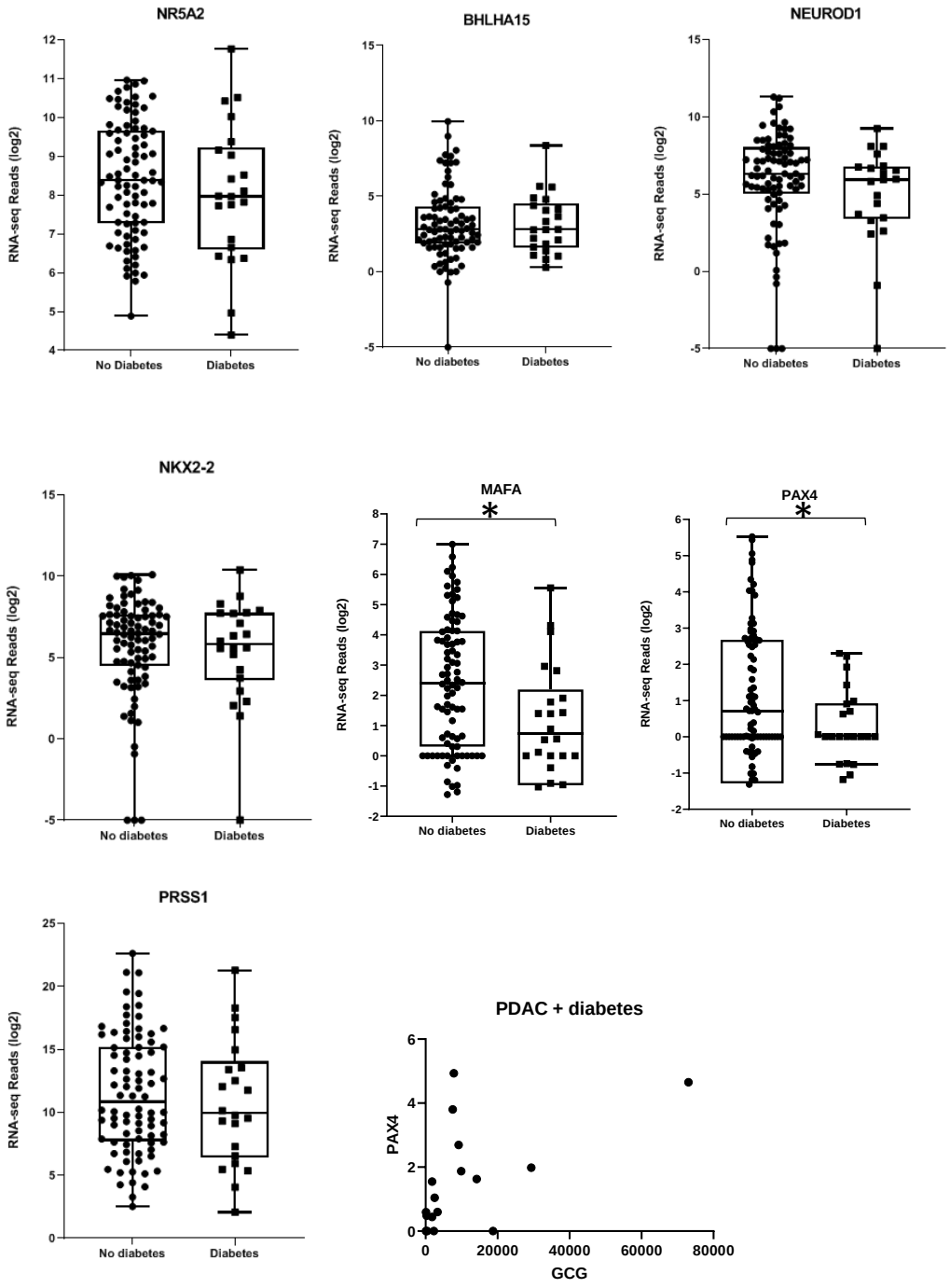


Figure 4-9 Gene expression analysis for the ADEX subtype. Gene expression of *MAFA* and *PAX4* was significantly reduced in the diabetic cohort. *PAX4* and *GCG* expression had a significant positive correlation (r = 0.65).

4.3.5 Leukocyte populations do not differ in the diabetic cohort

A recent study indicated a potential difference in the immune landscape between diabetics and non-diabetics with PDAC (Yan et al., 2017). I investigated this by conducting preliminary analysis using the CIBERSORT deconvolution algorithm to understand the immune cell populations in each cohort (Newman et al., 2015). This computational method is based on the differential expression of 547 genes to estimate the relative fraction of 22 leukocyte populations in each of the two cohorts of the TCGA PDAC samples. An additional group was added to the analysis to represent pancreatic samples with predicted higher levels of inflammation. Eight individuals with PDAC, diabetes and a history of chronic pancreatitis were compared with the other two cohorts. CIBERSORT analysis demonstrated that the diabetes cohort had a higher proportion of CD4 T cells compared to the non-diabetic cohort, but this was not statistically significant. No statistical significance was observed across the groups in any of the leukocyte populations (Figure 4-10).

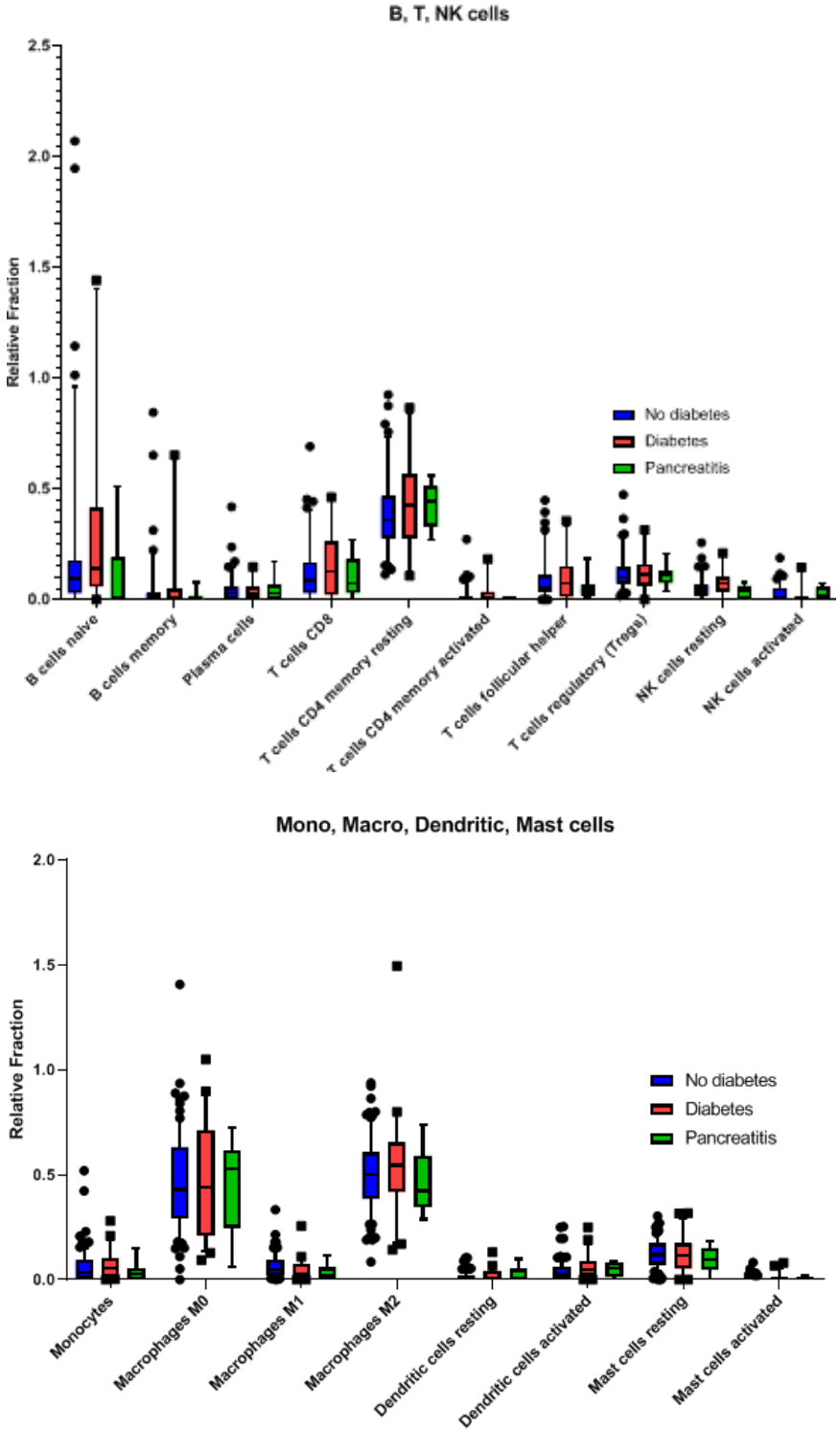


Figure 4-10 The relative populations of 22 leukocyte types present in the tumour microenvironment was determined by the CIBERSORT algorithm and compared between PDAC with diabetes, no diabetes and a history of pancreatitis. No statistical significance was observed but the PDAC with diabetes group had higher levels of CD4 T cell markers.

4.3.6 GO Pathway Analysis demonstrates that RNA expression levels in diabetic cohort are significantly associated with endocrine function in diabetes

To determine if the most significantly altered genes in the diabetic cohort were associated ($p<0.03$) with any particular gene pathways, I ran a GO Gene Pathway Analysis. After setting a cut-off of $p<0.03$, the programme used an unbiased analysis to identify pathways associated with βcell function and insulin synthesis (Table 4-5). This supports the clinical evidence demonstrating that the diabetic cohort has impaired islet function.

Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
Beta-Cell Death in Diabetes Mellitus Type 2	1.41E-07	7.03E-05	41.70940171	658.073963	NEUROD1;KCNJ11;ABCC8;IAPP;MAFA;INS
Proteins Involved in Epilepsy	5.44E-07	1.36E-04	3.783506994	54.57183142	SLC10A4;CHRNB2;KCNH2;GABRA1;GRIA2;KCNC1;DSCAM;KCNIP1;KCNJ11;RASGRF1;SLC4A10;ATP1A3;GRIK2;GABRG2;GJD2;NEUROD1;GRM3;HRH3;KCNQ2;CRH;BSN;SCN1A
Insulin Synthesis in beta-Cell	9.91E-07	1.65E-04	12.87553648	177.9991392	PCSK2;NEUROD1;KCNJ11;ABCC8;SCG5;IAPP;MAFA;INS
Neonatal Diabetes Mellitus	3.58E-06	3.61E-04	19.24358974	241.2984474	NEUROD1;KCNJ11;RFX6;ABCC8;GCK;INS
Insulin Secretion	5.02E-06	3.61E-04	17.86813187	218.0384427	NEUROD1;KCNJ11;ABCC8;GCG;GCK;INS
alpha-Cell to beta-Cell Interconversion (Hypothesis)	5.02E-06	3.61E-04	17.86813187	218.0384427	PCSK2;NEUROD1;PAX4;GCG;MYT1;INS
Ghrelin Influence on Insulin Secretion (Hypothesis)	5.05E-06	3.61E-04	29.72738349	362.5413458	GHSR;RASGRF1;GCG;INS;SSTR5
Proteins Involved in Neonatal Diabetes Mellitus	2.02E-05	0.0011516	41.53617021	448.9609706	KCNJ11;ABCC8;GCK;INS
beta-Cell Recovery in Diabetes Mellitus	2.07E-05	0.0011516	13.16261808	141.9455366	G6PC2;KCNJ11;ABCC8;GCGR;GCK;INS
Inherited Channelopathies	3.33E-05	0.0016232	11.90781441	122.7757817	CHRNB2;GABRA1;KCNJ11;KCNQ2;GABRG2;SCN1A
Maturity Onset Diabetes of the Young (MODY)	3.57E-05	0.0016232	33.22723404	340.2484732	NEUROD1;PAX4;GCK;INS
Hyperglycemia and Hyperlipidemia Trigger beta-Cell Apoptosis	4.15E-05	0.0017298	11.36596737	114.6763912	KCNJ11;EDEM1;FFAR1;GCK;ST18;INS
Insulin Release Inhibition by Somatostatins	1.28E-04	0.0049101	62.17834395	557.4978781	GCG;INS;SSTR5
Transcription Factors in beta-Cell Neogenesis (Rodent Model)	1.73E-04	0.0061797	11.55413409	100.0823787	NEUROD1;PAX4;MAFA;MYT1;INS

Table 4-5.

Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
Beta-Cell Granules Exocytosis	**2.14E-04**	**0.006698**	**10.94546067**	**92.46689819**	**RIMS2;UNC13A;KCNJ11;ABCC8;INS**
G-Proteins Signalling in Insulin Secretion in beta-Cell	2.14E-04	0.006698	10.94546067	92.46689819	GPR119;GCGR;RASGRF1;FFAR1;INS
Autoimmune Polyglandular Syndromes Progression (Hypothesis)	2.51E-04	0.0071709	41.45010616	343.6520044	PTPRN;MAFA;INS
Glutamate, D-Serine, and ATP Release from Astrocytes	2.58E-04	0.0071709	16.6093617	137.2258363	P2RY1;SLC17A6;SLC17A8;SYT7
Beta-Cell Dysfunction by Steroids in Diabetes Mellitus (Hypothesis)	4.31E-04	0.011348	31.08598726	240.8815628	G6PC2;KCNJ11;GCK
High Fat Diet Activates cAMP Related Exocytosis in Synapse and Endocrine Cell	6.39E-04	0.0159816	8.315991471	61.16573726	RIMS2;UNC13A;GCGR;RASGRF1;GCG
Wolfram Syndrome Progression (Hypothesis)	6.39E-04	0.0174277	11.86139818	85.63671077	KCNJ11;ABCC8;EDEM1;INS
Glutamate Overdose and Aura Effect in Familial Hemiplegic Migraine Type 3	6.39E-04	0.0206798	11.07007092	77.51447003	SLC17A6;SLC17A8;SYT7;SCN1A
MELAS Syndrome	9.99E-04	0.0217151	20.72186837	143.1645285	KCNJ11;ABCC8;INS
DRD1/3 -> Potassium Uptake	0.001401963	0.0292076	17.76069154	116.6856404	KCNJ6;KCNJ11;ABCC8
Proteins Involved in Diabetes Mellitus Type 2	0.001673886	0.0334777	3.545952526	22.6678823	NEUROD1;KCNJ11;ABCC8;GCGR;IAPP;GCG;FFAR1;GCK;INS
L-cell: GCG, PYY and 5-HT Release	0.001928675	0.0370899	8.737737962	54.61891942	PCSK2;GPR119;GCG;FFAR1

Table 4-5 Pathway analysis of the most significantly altered genes in diabetic cohort identify with pathways associated with β-cell function and insulin secretion.

4.4 Discussion

Individuals with long duration diabetes show an increased risk of developing cancers of the pancreas, liver, endometrium, colon, rectum, breast and bladder (Giovannucci et al., 2010, Maitra et al., 2018). From that list, the presence of diabetes is found in much higher frequency with pancreatic cancer than any other type of cancer. However, individuals diagnosed with diabetes after the age of 50 are also at risk of developing diabetes as a result of pancreatic cancer, particularly the progression of PDAC. This form of diabetes is thought to be a 'symptom of cancer' and may represent a potential window of opportunity for early diagnosis of PDAC, which would benefit survival outcome. Indeed, the National Cancer Institute (NCI) has acknowledged that studying the relationship between diabetes and PDAC is one of the highest research priorities in PDAC research (National Cancer Institute, 2014).

The aim of this chapter was to determine if a diagnosis of diabetes could influence the subtype of tumour present as described by Bailey et al. (2016). Pancreatic progenitor and ADEX subtypes are characterized by changes in expression of genes involved in endocrine cell fate, including genes associated with early progenitor cells and mature β-cells. *INS, GCG, SST, MAFA,* and *PAX4* were all significantly reduced in the diabetic cohort. While the diabetic cohort did not genetically match any tumour subtype, indicating that the pathology of diabetes did not influence categorization into a particular tumour phenotype, it cannot be ruled out that genes altered as a result of islet pathology from diabetes can affect this categorization. Conversely, a certain subtype may be associated with a higher risk of developing diabetes.

Interestingly, *INS,* GCG, and *SST* were all significantly reduced in the diabetic cohort. This may indicate loss of islets through destruction, or as the tissue

sections demonstrated, islet function may be impaired by high levels of fibrosis. PDAC fibrosis compromises drug delivery, limits accessibility of immune cells to the tumoral tissue and promotes disease aggression and chemoresistance (Pelosi et al., 2017). While the data collected in this study cannot determine if high levels of fibrosis are impairing islet function, there were no significant differences observed between the cohorts in gene expression of markers of fibrosis (collagen, elastin, fibronectin, fibrin). Indeed, when analyzing the tissue sections, the exocrine looked quite similar across both cohorts.

Single-cell RNA seq would provide greater clarity on the types of cells immediately surrounding the islets, plus any differences seen in the basement membrane of the islets across the two cohorts. Additionally, islet impairment from fibrosis and tumour-induced destruction of the endocrine cells are likely to occur within the tumour microenvironment, as opposed to the unaffected areas of the pancreas. This may alter the cross-talk occurring between exocrine and endocrine cells within that localised area, a theory which is also supported by the observation that removal of the tumor can restore normal glycemic control. The molecular crosstalk between the exocrine and endocrine pancreas in health and disease is complex and not fully understood. As described earlier, recent studies show a clear link between pathological conditions of the exocrine and endocrine pancreas. For instance, chronic pancreatitis is estimated to constitute 25–75% of the risk of diabetes, depending on the etiology of disease (Hart et al., 2016, Yatchenko et al., 2019).

The length of time since diabetes diagnosis (days vs. years) did not correlate with RNA expression levels of *INS, GCG,* and *SST.* This may suggest that the presence of the tumour, rather than the pathological mechanisms of diabetes are causing the downregulation of these endocrine genes. The preliminary Go

Gene Pathway analysis indicated that gene expression differences in the diabetic group were mainly those affecting β-cell function and insulin release pathways.

Clinically, PDAC associated with long-standing T2D and recent onset diabetes have similar presentations, and patients are given similar treatments to manage their diabetes. While the 'dual causality' debate between PDAC and diabetes continues, researchers have not investigated the possibility that PDAC may alter an existing form of diabetes within the tumor microenvironment. Researchers have assumed that the type of diabetes remains the same post-PDAC diagnosis, especially for patients with long-standing T2D, but the tumour may alter the form of diabetes, so all patients with PDAC and diabetes may have a similar form of T3cDM, at least in some areas of the pancreas. Until the mechanisms underlying T3cDM are fully investigated, and the molecular differences between T2D and T3cDM are better understood, this remains unknown. However, this concept is also supported by the observation that the functionality of islets is highly heterogenous (Dybala and Hara, 2019). Not all islets respond to stimuli or immune assault similarly, and indeed, even in cases when enterovirus infection is present, not all islets are affected (Richardson et al., 2013). The tumor microenvironment may only affect islets within close proximity to the neoplasia.

The reduction in *KRT19* in the diabetic group is of interest because *KRT19* has been reported as an indicator of prognosis, with higher expression levels associated with poor outcomes (Yao et al., 2016, Dugnani et al., 2018). This is contrary to a study that found long-standing diabetes with PDAC is associated with unfavorable pathology as well as worse postoperative and oncologic outcomes. However, these patients also presented with additional risk-factors

such as higher BMI that can also contribute to worse outcomes (Hank et al., 2020).

A recent study analysed PDAC donor cases with diabetes, including donor cases from the TCGA database, and *PDCD1* and *CTLA4* were shown to be upregulated in the cohort with diabetes, leading the authors to suggest that a clinical diagnosis of diabetes with PDAC may indicate immunosuppression. They also suggested that individuals with PDAC and diabetes should be managed separately to other PDAC patients on immunotherapy, as diabetes may affect the outcome of treatment (Yan et al., 2017). My study found that expression of *PDCD1* displays a binary pattern in the diabetic cohort, with a *PDCD1* high and *PDCD1* low group. The *PDCD1* high group may fall into the category of the immunogenic subtype of PDAC (Bailey et al., 2016), but overall, my results do not support the theory that individuals with PDAC and diabetes are immunosuppressed due to high *PDCD1* expression. Since I curated all samples to remove individuals with a history of chronic pancreatitis, plus any non-PDAC cancers (due to misclassification), this could indicate that pancreatitis may skew the observation that individuals with PDAC and diabetes are immunosuppressed. Yan et al. (2017) did not curate donor samples and therefore, mechanisms involved in pancreatitis and non-PDAC cancers may have influenced their results. Additionally, they found PD-1 expressed on the surface of pancreatic ductal adenocarcinoma cells (PANC-1) following cytokine treatment, but this runs contrary to the view that PD-1 is typically found on immune cells. In chapter three, I found PD-L1 to be expressed on the surface of PANC-1 cells, albeit in significantly lower amounts compared to EndoC-βH1 cells. The relatively low expression of PD-L1 on PANC-1 cells, and results from this chapter indicating that *PDCD1* expression did not correlate with *PDL1,* may

indicate a lack of expression of PD-L1 on tumour cells. This would support previous studies demonstrating that anti-PD-L1 immunotherapy has poor efficacy in treating PDAC compared to other cancers (Kabacaoglu et al., 2018).

Regulators of PD-L1 include IL-6, IFN-α, and IFN-γ, but only *IL6* seems to be upregulated in the tumour microenvironment within the diabetes cohort. *PDL1* does not positively correlate with *IFNG*. However, there is a very strong correlation between *PDL1* and *STAT1,* but not *STAT2*. Therefore, the regulation of PD-L1 in the microenvironment of PDAC may differ to the mechanisms explored in Chapter's two and three.

A limitation to this study is the analysis of RNA expression without confirming my findings at the protein level. It would be interesting to measure β-cell expression of key targets at the protein level from both cohorts.

I observed what appeared to be a thickening of the basement membrane around the islets in the tissue sections of the non-diabetic cohort with PDAC. This could potentially prove beneficial in a T1D setting as a mechanism to protect the islets from leukocyte infiltration and insulitis. The islet basement membrane functions as a physical barrier to leucocyte migration into islets and degradation of the islet basement membrane marks the onset of destructive autoimmune insulitis and diabetes development in NOD mice (Irving-Rodgers et al., 2008). It may be possible to recreate the thickening process in the laboratory in future studies using PDAC tissue explants or organoids in a microfluidic culture system *ex vivo*.

In summary, this chapter demonstrated the following:

1) That diabetes is not associated with a particular subtype of PDAC, but markers associated with the pathology of diabetes (reduced *INS*, *GCG*,

SST, *MAFA* and *PAX4*) may potentially result in a patient tumour being misclassified. For example, the ADEX subtype is characterised by upregulation of transcriptional networks that characterize both exocrine and endocrine lineages at later stages, rather than one or the other as is the case in normal pancreas development (e.g. INS, NEUROD1, NKX2-2 and MAFA). In the diabetic cohort, *INS* and *MAFA* are reduced and this is likely a marker of the diabetes, as opposed to tumour subtype.

2) Contrary to Yan et al. (2017), the immunogenic subtype is not derived from PDAC individuals with diabetes, and this cohort is not immunosuppressed due to upregulation of *PDCD1* and *CTLA4.* Therefore, my study does not support the notion that patients with diabetes and PDAC should be managed separately for immunotherapy due to potential differences in efficacy of anti-PD-1 or anti-PD-L1 immunotherapy.
3) Chronic pancreatitis is an independent risk factor for development of T2D as well as pancreatic cancer and is associated with germline mutations. Caution should be taken when investigating PDAC associated T3cDM and donor cases should be curated so results do not reflect the processes of pancreatitis. For example, we currently do not understand how T2D differs to pancreatitis linked diabetes and diabetes triggered by tumour growth.
4) The basement membrane of islets contained within the area of the neoplasm can become unusually thick. The relevance for this remains unknown, and there is scope for further research.

Chapter 5

5 Epigenetic characterisation of a persistent Coxsackie virus infection in pancreatic cells

5.1 Introduction

Clinical and epidemiological studies in humans have long supported a role for viral infections as environmental triggers for the development of T1D (Richardson et al., 2014b, Hyöty, 2016, Rewers and Ludvigsson, 2016, Vehik et al., 2019). Our team has previously determined that enterovirus infection can be detected in autopsy pancreas specimens recovered from of recent-onset T1D patients. These studies revealed that a presence of viral antigens and inflammation within insulin-containing islets could be detected at much higher frequency than in older individuals with T2D or young people with no diabetes (Richardson et al., 2013). Enteroviruses have also been implicated in the development of T1D from prospective birth cohort studies and were detected within the pancreas before the onset of β-cell damage, suggesting a potential role for viruses in promoting the conditions required for immune assault (Laitinen et al., 2014, Oikarinen et al., 2014, Sioofy-Khojine et al., 2018).

Coxsackie viruses are small, non-enveloped viruses with positive-sense single-stranded RNA genomes. They can be separated into two groups, A and B, based on their pathogenesis in newborn mice (Coxsackievirus A results in muscle injury, paralysis, and death; Coxsackievirus B results in organ damage but less severe outcomes). There are six different serotypes of Coxsackie B virus (CBV1-6) which are distinct in their antigenic and genetic properties (Tuthill et al., 2010). To date, two serotypes have been associated with increased risk to T1D (CBV1 and CBV4) but several serotypes have been tested *in vitro* for their pathogenic impacts on pancreatic cells (Laitinen et al., 2014).

Evidence from clinical studies identified antibody responses to selected CBV serotypes in patients with T1D (Oikarinen et al., 2014, Laitinen et al., 2014), and *in vitro* and *in vivo* studies have demonstrated that CBVs can efficiently infect β-cells, trigger innate immune responses, cause cellular dysfunction and impair insulin secretion (Gallagher et al., 2015, Sarmiento et al., 2017, Op de Beeck and Eizirik, 2016). Moreover, infections can induce inflammation, stress responses and β-cell death, which could lead to the triggering of chronic islet autoimmunity in genetically predisposed individuals (Morgan et al., 2014). Interestingly, single nucleotide polymorphisms (SNPs) located within or close to genes involved in cellular antiviral responses are also important risk factors for T1D (ex. MDA5, Tyk2) (Op de Beeck and Eizirik, 2016, Lincez et al., 2015, Marroqui et al., 2015, Izumi et al., 2015).

The extent to which CBVs can directly cause β-cell destruction in an interesting question. For example, it might be argued that a 'perfect storm' of conditions is required to trigger T1D in children, otherwise all children infected with CBVs would develop the disease. Certain serotypes of CBV may also be more important than others in determining β-cell fate. Dotta et al. (2007) reported that CBV4 infected human islets from patients with T1D had increased expression of cytokines IL-10 and TNF-α but not IFN-γ, a cytokine more associated with β-cell destruction. Evidence of β-cell dysfunction and impaired insulin secretory function was observed, but there was no indication of β-cell destruction. Interestingly, the authors describe how the infection *in vitro* caused a dysregulated insulin response to high glucose, but this occurred despite the presence of preserved insulin and proinsulin content in human islet cells. This

could potentially suggest that in the absence of concurrent autoimmunity, β-cell destruction is unlikely in the face of CBV infection. In 2016, a separate group studied CBV infection in human islets and recorded decreased *INS* gene expression followed by a negative impact on insulin granule release (Hodik et al., 2016).This, at least, supports a consistent hypothesis that CBV infection can negatively impact insulin responses, however it should be noted that the above studies involved acute infection models.

As discussed in the Introduction (**Chapter one**), large-scale studies such as the TEDDY study have demonstrated links between persistent enteroviral infection and development of T1D in young children. A common plausible hypothesis is that persistent (or chronic) CBV infection within the β-cells leads to cellular damage, either through direct anti-viral pathways in the cell or via virus-induced inflammation (Op de Beeck and Eizirik, 2016, Sane et al., 2013, Tracy et al., 2015, Richardson et al., 2013). Persistent CVB4 infection in the ductal pancreatic cell line, PANC-1, has been shown to result in the downregulation of PDX1, a transcription factor involved in the development and function of endocrine cells (Sane et al., 2013), and in the dysregulation of microRNAs that may potentially target T1D genes (Engelmann et al., 2017). Given that ductal cells may represent a reservoir from which new β-cells are differentiated, CBV infection could perturb this process leading to a depletion of the β-cell population during early development.

Work by Chapman and colleagues has shown that CBV can undergo a 5’ terminal deletion within its genome in the mouse pancreas and human cardiomyocytes (Chapman et al., 2008). This deletion allows the virus to adapt

to its host and persist intracellularly to avoid elimination by neutralising antibodies. After inoculation of NOD diabetic mice with CBV, viral RNA persisted in the absence of cytopathic virus in the pancreas for many weeks following the acute infectious period. Analysis of viral genomic 5' termini by RT-PCR showed CBV-terminal deletion populations displaced the parental population during persistent infection (Kim et al., 2008, Tracy et al., 2015). The available evidence implicating viral infection in pancreata from the Network of Pancreatic Organ Donors with Diabetes (nPOD) collection indicates that persistent infections could be potentially associated with the development of an enterovirus that contains a genomic terminal deletion. However, work is ongoing to confirm this observation in the nPOD collection with RNA sequence analysis, advanced fluorescence *in situ* hybridisation (FISH) and advanced proteomics.

As pathogens, all viral families possess extraordinary adaptive abilities and display rates of nucleic acid sequence change that are far higher than those occurring in the host cell they infect. Viral adaptation to the host can be described as watching evolution in real time as the viruses acquire antiviral drug resistance, mediate immune evasion or adapt to new cell culture conditions, receptors and hosts. Emerging evidence in non-pancreatic models suggests that persistent CBV infection can be established and may be responsible for chronic inflammation within target organs. Latent states and episodic reactivation may also contribute to the disease process (Feuer et al., 2002, Ruller et al., 2012, Feuer and Whitton, 2008, Sin et al., 2015).

Previous experiments have demonstrated that HLA class I presence is elevated in the majority of cells persistently infected with CBV viruses, consistent with

observations made in the islets in T1D pancreas. Others have further confirmed that HLA-A, B2M, STAT1 and MxA are upregulated at the RNA level in the persistently-infected cells compared to mock-infected cells, which also accords with observations made in the T1D pancreas (Richardson et al., 2016, Domsgen et al., 2016, Nyalwidhe et al., 2017). When looking at environmental events prior to development of T1D, the persistent model of infection allows us to assess the impact of a persistent infection on pancreatic function and viability.

Our group has previously demonstrated that plaque morphology and the cytopathic effects of viral infection are altered during the period in which the virus transitions from an acute to a persistent state. These responses were observed in both the PANC-1 and 1.1B4 cell lines. This further supports the suggestion that the virus undergoes genetic alteration to sustain a persistent infection, a characteristic observed in several other types of virus (Feuer et al., 2002). Identification of these changes would be crucial for the understanding of the mechanisms of chronic infection.

A recent study on persistent CBV infection in pancreatic cells demonstrated how CBV persistence modifies the proteome and secretome of PANC-1 cells, and alters pathways associated with mitochondrial metabolism, viral receptor signalling and regulation of secretory pathways (Lietzén et al., 2019). Therefore, this chapter sought to investigate the properties of CBV during the emergence of a persistent infection by looking at the methylation signatures and epigenetic changes that occur as a result of the virus establishing a persistent infection in PANC-1 cells and comparing those found in acute infection settings. This allows

us to further understand how the cellular landscape is changing as a result of the virus hijacking the cellular networks and adapting to its host to survive. It is of significant interest to understand the replication potential of viruses associated with T1D and to determine the extent to which persistent infections cause cell stress and altered gene expression in cell survival pathways and cytokine expression. The identification of the nature of virus infection, the development of reliable tools to characterise viral infection in the T1D pancreas and finally the identification of specific viruses is important for early therapy/vaccines for T1D.

This Chapter represents an exploratory study and focuses on the differences between acute and persistent infection through the analysis of methylation changes in the host cell. Previous research has focused on viral presence versus non-viral presence (i.e. mock infection) in the pancreas, but this chapter introduces another variable, namely the timing of infection. I have normalised the data to account for changes in infection versus non-infection, but then looked for changes that occur as a result of the time taken to move between acute and persistent states.

Proving the hypothesis that persistent infection can alter β-cell function requires the development of adequate models. Human islets are comprised of primary cells and therefore do not proliferate in culture, and there is extensive cell lysis in the outer mantle of islets cells exposed to enteroviruses *in vitro* (Richardson et al., 2014a). To progress these studies, cultured human β-cell models would be ideal and 1.1B4 cells would have been productive hosts for persistent coxsackie infections. However, as outlined in chapter two the 1.1B4 cell line is

no longer a viable option, therefore PANC-1 cells were chosen to represent the most suitable alternative for tracking persistent infections and to gain an improved understanding of the co-evolutionary processes between host and pathogen.

5.2 Epigenetics

The term ‘epi’ means above, therefore, epigenetics is the study of information coded above the standard genetic code. Epigenetics investigates the alterations in gene expression, which result from heritable but potentially reversible changes in chromatin structure and/or DNA modifications (Henikoff et al. 1997). Cells are not physiologically identical, even if they contain the same genetic information. This make sense from an evolutionary perspective, because cells must be able to adapt to varying environmental conditions. Plasticity without permanent changes to the genetic code can occur through epigenetic mechanisms that regulate gene expression. However, such changes can also cause disease, as seen in various forms of cancer (Jones et al. 1999, Feinberg et al. 2004, Laird 2005, Esteller 2008, Rauch et al. 2012).

The most recognised form of epigenetic change occurs through DNA methylation of mammalian genomes, which then modulates the transcription of specific genes. This is well-studied because there are archived DNA resources readily available for interrogation (Lunnon et al. 2013). The methylation of cytosine in CpG dinucleotides, forming 5-methylcytosine (5mC), can disrupt the cell’s transcriptional machinery by blocking the binding of transcription factors and attracting methyl-binding proteins that initiate chromatin compaction and bring about gene silencing (Klose et al. 2006). The predominant focus to date has been methylation within CpG Islands (CGIs), dense regions of CpG sites,

located within the 5' promoters of many constitutively expressed housekeeping control genes. However, recent data suggest that the relationship between 5mC and transcription may be more complex, with gene body methylation and non-CpG methylation often being associated with active gene expression (Hellman et al. 2007, Ball et al. 2009, Lister et al. 2009, Rauch et al. 2009, Aran et al. 2011) and alternative splicing (Lyko et al. 2010, Flores et al. 2012). Further research has now demonstrated that other cytosine modifications may also have functional roles in gene regulation (Inoue et al. 2011, Ito et al. 2011) and include 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). These modifications occur as stages in the demethylation of cytosine (Breiling et al. 2015). However, this chapter will predominantly focus on 5mC modifications and has sought to identify the differentially methylated positions associated with persistent CBV infection of PANC-1 cells.

5.3 Methods

5.3.1 Propagation of enterovirus stocks

HEp-2 cells were infected with Coxsackie virus strain CBV4.E2 (kindly provided by Prof. Didier Hober, Lille University, France (Benkahla et al., 2019)). The cells were incubated for 3-4 days until most had detached from the substrate. The culture medium was not changed during this time. Following this period, the cultures were frozen and thawed 3 times then centrifuged (1000 x g; 10 min) to clear the lysate prior to aliquots being stored at -80°C. The viral titre was estimated using a standard plaque assay.

5.3.2 Plaque Assay

HEp-2 cells were cultured in a 6-well culture plate as confluent monolayers at a density of 10^6 cells/well. After 24 hrs, medium was removed and cells were

washed once with PBS and overlaid with 2 mL of dilutions of the virus suspension, and then incubated at 37°C for 2 hrs. After removal of the supernatant, cells were overlaid with 3 ml of agar containing Eagle's minimal essential medium (MEM). Three days later, the cells were fixed with 10% PFA for 2 hrs. After removal of PFA and agar, cells were stained with 1% crystal violet for 30 min. After washing and drying the plaques were observed and counted (Alidjinou et al., 2017).

5.3.3 Infection in PANC-1 cells with CVB4.E2

PANC-1 cells were seeded at 60% confluency then incubated for 18-24 hrs prior to infection with CVB4.E2, after dilution of the virus stocks in media to achieve an MOI of 0.01 or 0.1. Mock infected cells received vehicle alone. The mock and infected cell lines were maintained and passaged independently but in parallel 24 hrs post infection, the cells were washed in cold PBS and fresh medium added. Cells were maintained until day 3 post infection when initial samples were collected. Cells to be propagated further were scraped from the substrate, gently re-suspended in fresh medium then seeded into culture flasks at 15-25% confluency (mock infected) or 40-70% confluency (CVB infected cells) and cultured as appropriate. Samples representing acute infection were extracted on day 7 and those representing persistent infection on day 76.

Viral titre was determined by plaque assay (performed in duplicate) for n=3 per condition.

5.3.4 DNA Extraction

All plasticware used for DNA extraction was sterile and DNase-free, and surfaces were cleaned using ethanol and/or Alconox (Sigma-Aldrich, cat no.: 242985) to remove contaminants and DNAses present. DNA extraction of CBV

infected and mock infected PANC-1 cells was done using a standard phenol-chloroform method. This method has been widely utilized for EWAS as it provides sufficient yields of high-quality DNA for downstream purposes. The protocol was modified from the phenol chloroform method developed by Sambrook and Russell (2006).

5.3.5 Bisulfite treatment of DNA

Bisulfite treatment is a method that provides a base pair level sensitivity measure for DNA modifications (including 5mC). In this process unmodified cytosine is converted to uracil, but relevant modifications to cytosine including 5-mC and 5-hmC render these bases unreactive. The sequence can then be amplified via subsequent PCR, resulting in all uracil and thymine residues being amplified as thymine and only modified cytosine residues being amplified as cytosine. Bisulfite treatment using the Zymo EZ-96 DNA Methylation-GoldTM Kit ideally utilizes 500ng high quality DNA in 20µl to provide sufficient bisulfite treated material for cytosine modification array profiling and subsequent validation experiments. The manufacturer's protocol is available from http://www.zymoresearch.com/downloads/dl/file/id/59/d5007i.pdf.

5.3.6 Methylomic profiling

5.3.6.1 Illumina Infinium Methylation BeadChip Array

All samples were processed using the Illumina Infinium Human Methylation EPIC Beadchip Array (Illumina Inc., CA, USA) according to the manufacturer's instructions, with minor amendments.Data were quantified using the Illumina HiScan System (Illumina, CA, USA). The Infinium methylation EPIC array allows the interrogation of methylation patterns at a genome-wide level, covering more than 850,000 methylation sites. This array is an ideal solution for epigenome-wide association studies.

The Infinium HD Methylation Assay combines bisulfite-converted DNA and whole-genome amplification with direct, array-based capture and scoring of the CpG loci. Illumina iScan or HiScan system measures the signal intensity to generate beta values, a measure of the degree of 5-mC at each locus (beta value represents: 0-methylated, 1-fully methylated). Allele-specific single base extension of the probes incorporates a labelled nucleotide. Signal amplification of the incorporated label further improves the overall signal-to-noise ratio of the assay.

A denaturing and neutralization stage occurs before the bisulfite-treated DNA is amplified across the whole genome. The amplified products are then fragmented using endpoint fragmentation to prevent over fragmentation. Isoproponal precipitation is followed by centrifugation at 4°C to collect the fragmented DNA.

The precipitated DNA is suspended in hybridization buffer and DNA samples dispensed onto BeadChip arrays and incubated in an Illumina Hybridization Oven to hybridize the samples onto the BeadChip array surface. DNA samples anneal to locus-specific sites on the BeadChip array and bound probes stop one base before the locus of interest. Un-hybridized and non-specifically hybridized DNA are washed away and the BeadChip array then undergoes extension and staining in capillary flow-through chambers. Single-base extension of the oligonucleotides on the BeadChip array, using the captured DNA as a template, incorporates detectable labels on the BeadChip array and determines the 5-mC level of the specific CpG sites. The intensity of the signal informs the allelic ratio at that locus. Finally, the BeadChip array is imaged on the Illumina HiScan or iScan System, using a laser to excite the fluorophore of

the single-base extension product on the beads. The scanner records high resolution images of the light emitted from the fluorophores. These in turn are used by the genome studio® software to produce a β- value at each locus. A full description of this methodology can be found on the Illumina website http://www.illumina.com/products/by-type/microarray-kits/infinium-methylation-epic.html.

5.3.7 Data analysis

5.3.7.1 Data quality control

All computations and statistical analyses were performed using R 3.3.2 (R Development Core Team 2012) and Bioconductor 3.5 (Gentleman et al. 2004). Signal intensities were imported into R using the methylumi package (Davis et al. 2012) as a methylumi object. Initial QC checks were performed using functions in the methylumi package.

5.3.7.2 Data normalisation and quality control

The ratio of the intensity of the fluorescent signal for M (modified) and U (unmodified) was calculated to determine the DNA modification level of each CpG site. This gives a beta (estimate of DNA methylation level) value for each site ranging from 0 (*i.e.* all cytosines at that site are unmodified) to 1 (*i.e.* all cytosines at that site are modified). The epigenetics teams at Essex University and Exeter University have developed the *wateRmelon* package in R 3.3.2 (Vienna 2012, Pidsley et al. 2013), which offers a range of normalisation function and tools that were used to pre-process and normalise the DNA modification data presented in this chapter.

5.3.7.3 Data quality control

All computations and statistical analyses were performed using R 3.3.2 (R Development Core Team 2012) and Bioconductor 3.5 (Gentleman et al. 2004).

Signal intensities were imported into R using the methylumi package (Davis et al. 2012) as a methylumi object. Initial QC checks were performed using functions in the methylumi package.

The presence of SNP variation in close proximity to the query CpG site can be an issue with the probe design for the Illumina array. The confidence in the 5mC data at these sites could be low due to the presence of these polymorphisms (Chen et al. 2013, Price et al. 2013). Therefore, the DNA modification levels detected by the probes could simply be a representation of the underlying genetic polymorphisms. Additionally, 6-8% of the probes in the array have been found to cross-hybridise with other genomic locations and therefore do not accurately estimate the 5mC levels at the annotated site (Chen et al. 2013, Price et al. 2013). As a result of these issues, probes known to either cross-hybridise with another site or contain SNP variation were excluded prior to further analysis of the data presented in this chapter.

5.3.7.4 Linear regression analysis

The effects of time and infection were regressed for the analysis. For the identification of differentially methylated probes altered with respect to infection stage, quantitative analysis was performed with respect to infectious stage using a linear regression model. Linear regression was performed using binary factors (0 and 1) to represent both time (i.e. acute = 0, persistent = 1) and control versus infection case (i.e. control = 0, infection = 1). Mock samples were analysed separately and resulting significant probes removed from the significant probe list from the infected cases. This ensured the significant probes identified were specific to acute versus persistent infection and not a natural epigenomic transition due to cellular age. This also provided information

on the epigenomic transitions occurring as infection persists, giving insight into the potential shifts in gene expression over time.

Significant differentially methylated probes were identified between cases by p value, a value of the probability that observed differences are by chance, thus, probes with relatively lower p values are likely to be affected by the ongoing presence of infection rather than by random intercellular variation.Q-Q plots were assessed to check for statistical inflation.

5.3.7.5 Pathway analysis

Pathway analysis of DMPs was performed using the *missmethyl 1.10.0* (Phipson et al. 2016) package from Bioconductor. Pathways were included if they met the following criteria: firstly the number of genes in the gene list was greater than one and less than 2000, and secondly more than one gene from our analysis was present in the pathway.

5.4 Results

5.4.1 Establishing persistent Coxsackievirus infections in PANC-1 cells

To complete the aims of this Chapter, I first needed to establish a pancreatic model of persistent infection. Human pancreatic β-cell lines like Endo-βCH1 cannot easily sustain persistent infections with CBV4E.2 as extensive cell death occurs shortly after initial infection. The prolonged doubling time of this cell line also make infection studies difficult, therefore, I chose to use the PANC-1 cell line.

PANC-1 cells were infected with CBV4E.2 in T25 flasks at an MOI of 0.1 and 0.01. Cells underwent an acute response to the infection as seen by the early increase in cell death (Figure 4-1A) followed by the development of a persistent infection phase where cell death decreased. Figure 4-2 demonstrates the

expected profile of cell death during the development of a persistent infection. The acute phase is characterised by high rates of viral replication and increased viral protein and RNA production. Increases in cytopathic effect (CPE), which occurs when the infecting virus causes lysis of the host cell or the host cell dies because of post-infection effects, are also observed. When faced with external viral invasion cells will deploy anti-viral defences and activate acute phase responses that include viral-RNA triggered defensive pathways and the production of inflammatory cytokines. Some of these signals lead to cell death as a way of controlling the viral spread, while others are used to warn neighbouring cells and to attract immune cells to destroy the invading virus. As the virus adapts to its host however, it alters its manipulation of the host cell to enter a period of slow replication and avoidance of host defence responses in order to survive.

To quantify the infectious virus and demonstrate a reduction in viral replication over time, I collected supernatant every 7 days from both mock and infected samples and conducted plaque assays. This method has been a reliable determination of viral titre for many viruses for ~70 years (Dulbecco and Vogt, 1953). Figure 4-3A demonstrates plaques formed by CBV4E.2 on a monolayer of Hep-2 cells. In this figure, the cells have been stained with crystal violet and the plaques are readily visible where the cells have been destroyed by viral infection. To minimise error, only wells containing between 10-100 plaques were counted and samples run in duplicate for each of the three experimental samples per condition (MOI 0.01, 0.1 and corresponding mocks) (Baer and Kehn-Hall, 2014). The decrease in viral titre over time is shown in Figure 4-3B. By day 40, the virus had begun to adapt to its host environment and to establish

a persistent infection. The persistence phase is demonstrated by the plateau of viral titre. Both viral titres tested (MOI 0.01 and 0.1) allowed for the establishment of a persistent infection although. Infection at MOI 0.1 reached a state of persistence earlier than MOI 0.01 because there was a lag in peak points during the acute phase.

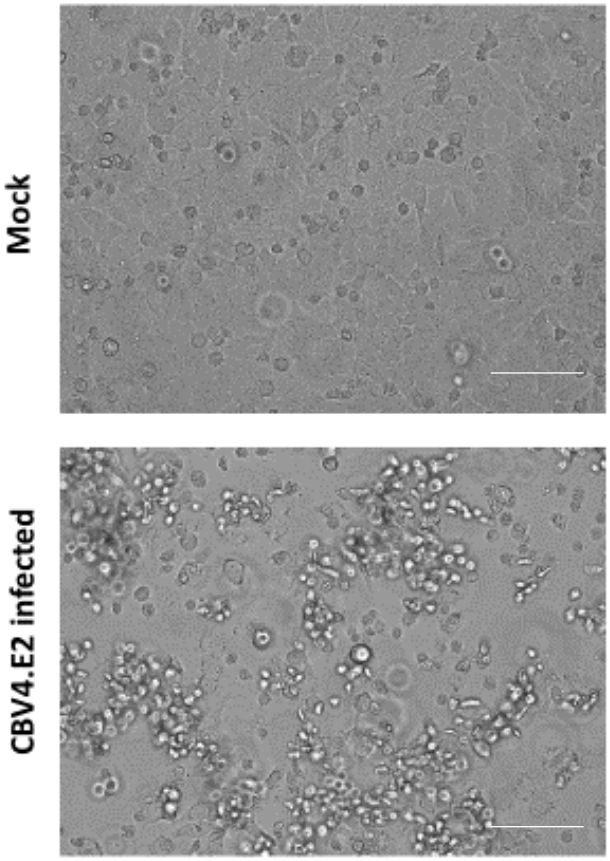


Figure 5-1 Acute infection of PANC-1 cells with CBV4.E2.

Infection of PANC-1 cells with CBV4.E2 causes a high level of cell death during the acute stages of infection. Scale bar 30 µm.

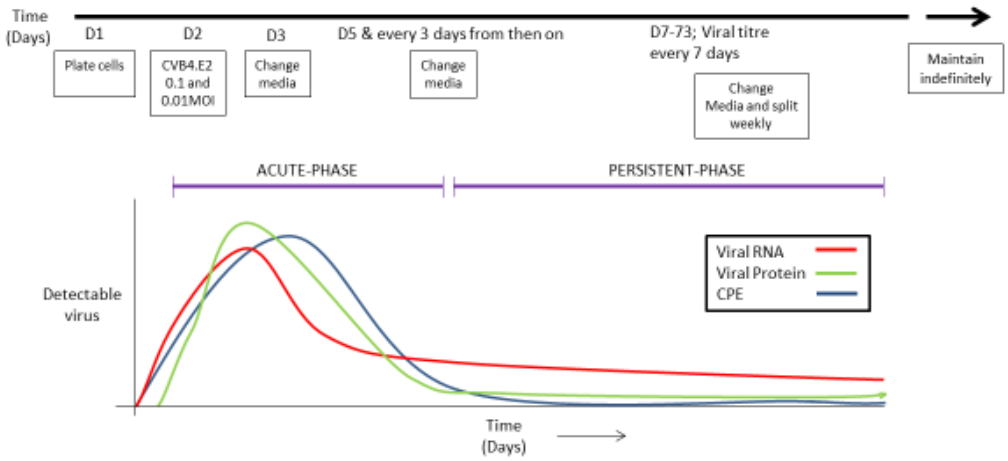
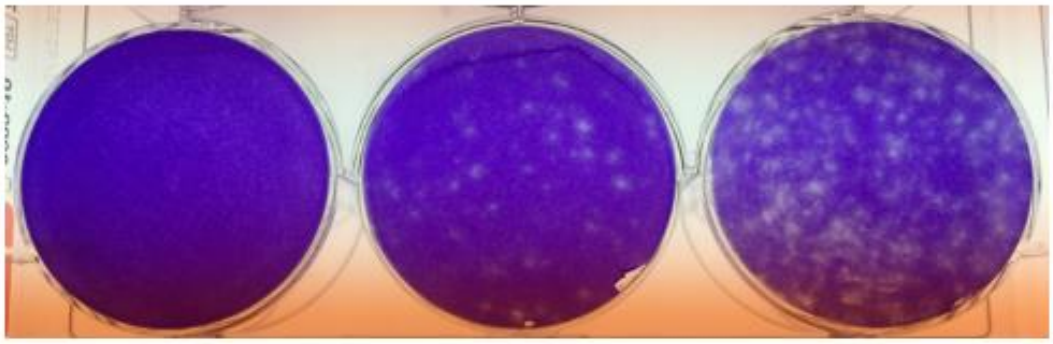


Figure 5-2 Infection kinetics of coxsackie virus.

Upon infection with virus, an acute phase is characterised by high rates of viral RNA, protein and cytopathic effect as a result of viral replication. As the virus adapts to the host cell, a persistent infection takes hold and viral RNA and protein levels remain relatively stable.

A



B

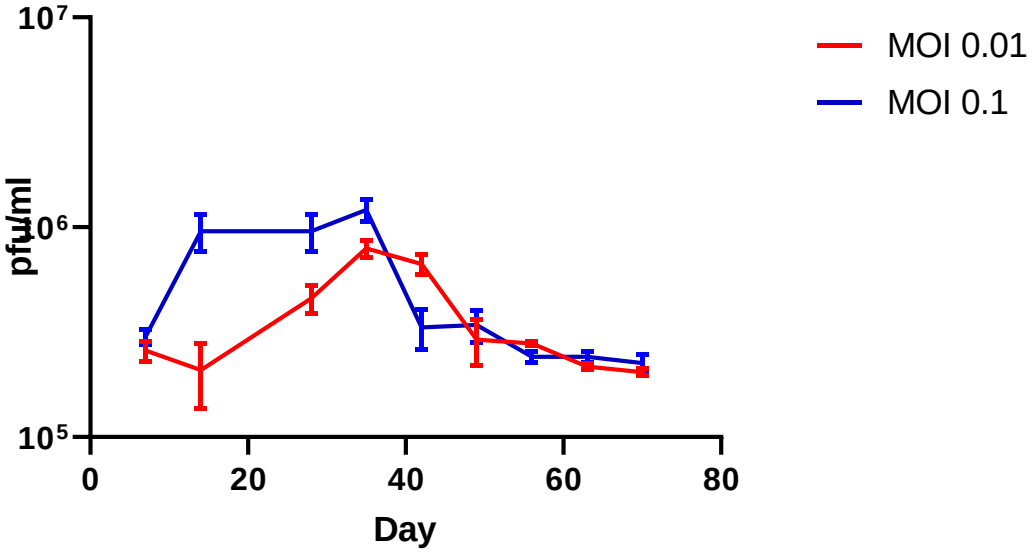


Figure 5-3 Establishing a persistent CBV4.E2 infection in PANC-1 cells.

(A) Representative example of plaque assays demonstrating an increase in cytopathic effects by around day 20 of the infection cycle (MOI 0.1). (B) Viral titres demonstrating a reduction in the quantity of virus produced post day 40 as virus establishes persistence n=3.

5.4.2 DMPs occur in PANC-1 cells as the virus adapts to its host and establishes a persistent infection

I wanted to identify loci that showed persistent infection-associated differential 5mC in the PANC-1 cell (Table 4-1). The top 100 genes displayed reached the significance level ($p<0.005$) and represent genes methylated as the virus established a persistent infection in PANC-1 cells. Linear regression modelling was conducted for the infected and non-infected groups, and the persistently and acute infected groups to reflect time and infectious state. This meant that any significantly methylated positions that resulted from time, rather than the infection state, were controlled for. No DMPs reached Bonferroni level of significance but as this chapter was an exploratory study, the top 100 genes are displayed. With thanks to Adam Smith, Jenny Imm and Lachlan MacBean for performing the Illumina Infinium Methylation BeadChip Array.

5.4.3 Persistent infection-associated DMPs in the EC feature in key biological pathways

To understand the potential effects these DMPs may be having on the biological processes within the PANC-1 cells, pathway analysis was performed on the top 100 genes annotated to DMPs in the cells that reached $p<0.005$. Using the gene ontology resource for visualising gene ontology terms (link to access: http://revigo.irb.hr/) a computational model of biological systems was run. None of the pathways reached significance (FDR $p<0.05$) but as this chapter represented exploratory work, all pathways are presented (Figure 4-4AB).

Pathways of interest include ‘interspecies interaction between organisms’, ‘cellular protein catabolism’ and various signalling pathways that involve ion channels, Ca^{2+} transport, mitochondrial processes and metabolic processes.

I thank Adam Smith and Lachlan MacBean for performing the pathway analysis.

#	Probe	Gene Annotation	#	Probe	Gene Annotation
1	cg00034019	TAF8 (-33)	**26**	cg05058516	C4orf46 (-34)
2	cg00288494	PACSIN3 (-8103)	**27**	cg05192589	ATP6V0B (+161)
3	cg00538248	PTPRT (+1361)	**28**	cg05597008	CHCHD10 (+500)
4	cg00615811	KCTD5 (+16)	**29**	cg06158944	RAP1GDS1 (-246)
5	cg00731913	XRCC6 (+152)	**30**	cg06555155	TMEM181 (+23974)
6	cg00753142	RCC2 (-37939)	**31**	cg06621094	KLF12 (-580778)
7	cg01013857	ENSG00000257743 (-23399)	**32**	cg07180832	FBXO17 (-11)
8	cg01076970	BTNL9 (-11976)	**33**	cg07293584	ANO1 (-92362)
9	cg01128603	SF3B2 (+585)	**34**	cg07714534	C11orf82 (-176)
10	cg01663665	CST7 (-132646)	**35**	cg08446032	OPRK1 (-100714)
11	cg01700096	SIX5 (-171)	**36**	cg08472341	BASP1 (+452807)
12	cg01833398	TARBP2 (+51)	**37**	cg08942080	FAM101A (+47859)
13	cg02709390	TARM1 (+3627)	**38**	cg09414137	JUN (+2192)
14	cg02715833	PPP1R3E (+1268)	**39**	cg09450550	LRRC37A3 (-3535)
15	cg02971902	RBMX (-29044)	**40**	cg09754868	SPEN (+1514)
16	cg03064241	RNF34 (-193)	**41**	cg09856153	UVSSA (-10167)
17	cg03269449	RER1 (-150)	**42**	cg10495460	PYY (+9885)
18	cg03453210	DCP2 (-34)	**43**	cg11141561	CAMLG (+214)
19	cg04168939	PACS2 (-13681)	**44**	cg11323020	C2CD2L (+99)
20	cg04184946	AKTIP (-98)	**45**	cg11662224	PRKCG (-50)
21	cg04203084	QRICH1 (+257)	**46**	cg11721100	MAP7D2 (+688)
22	cg04215055	MGAT5B (+42792)	**47**	cg11750133	FGFBP3 (+22022)
23	cg04372942	GPRC5C (-118)	**48**	cg11787907	CAMKK2 (+1827)
24	cg04456085	BMS1 (-38461)	**49**	cg11850639	PDE4A (+3723)
25	cg04806553	CDKN1A (+5508)	**50**	cg12323105	PMAIP1 (+9673)

Table 5-1.

#	Probe	Gene Annotation	#	Probe	Gene Annotation
51	cg05058516	CCNJ (+86216)	**76**	cg21092303	KCTD7 (-246975)
52	cg05192589	NCBP1 (+181)	**77**	cg21367232	SCAMP2 (-595)
53	cg05597008	KIF7 (-4)	**78**	cg21444693	ZNF418 (-48)
54	cg06158944	RNF6 (+698)	**79**	cg21755633	RAB1A (-524)
55	cg06555155	NUP210 (+216115)	**80**	cg21798060	CFB (+1525)
56	cg06621094	NRXN3 (+625054)	**81**	cg22704399	AMZ1 (+42512)
57	cg07180832	NCOA7 (-332)	**82**	cg22758021	UCK2 (+339196)
58	cg07293584	GHITM (-714139)	**83**	cg22818988	TRNT1 (-90)
59	cg07714534	HSF1 (+184)	**84**	cg22935477	UHRF2 (+269)
60	cg08446032	EPHX2 (+355)	**85**	cg22947154	HSPA2 (-362)
61	cg08472341	VIPR1 (-13)	**86**	cg23104539	GRB10 (-61486)
62	cg08942080	OFCC1 (+63519)	**87**	cg23173463	CDH11 (+643886)
63	cg09414137	BSPRY (-8708)	**88**	cg23300897	USP20 (+437)
64	cg09450550	WNT10A (+16543)	**89**	cg23338977	TMEM256 (-1488)
65	cg09754868	ZNF546 (-85)	**90**	cg23576358	S100P (-19282)
66	cg09856153	SLC9A7 (-141)	**91**	cg24221490	DENND5B (+587)
67	cg10495460	MLX (-214)	**92**	cg25017988	ENSG00000203546 (+37443)
68	cg11141561	DIAPH3 (-370)	**93**	cg25487370	RAPGEF2 (-163800)
69	cg11323020	ZNF324B (-193)	**94**	cg26095210	ZNF345 (+505)
70	cg11662224	KIAA1143 (-1)	**95**	cg26243475	RNASEH2B (+196)
71	cg11721100	MRPL43 (+256)	**96**	cg26246138	SCML2 (+234)
72	cg11750133	FARP1 (-3782)	**97**	cg26933422	MGAT1 (-1182)
73	cg11787907	CLTC (-247)	**98**	cg27100814	CGGBP1 (-1727)
74	cg11850639	CYR61 (-2648)	**99**	cg27238852	C16orf80 (-28821)
75	cg12323105	PTBP1 (-7416)	**100**	ch.1.4690234F	LGALS8 (+27309)

Table 5-1 Top 100 significant persistent infection-associated differentially methylated positions (DMPs) in the PANC-1 cells. Shown for each probe are GREAT annotation with brackets denoting number of base pairs distance from methylation site. Corrected for mock and time and linear regression derived p value between persistent and acute samples. All values $p<0.05$.

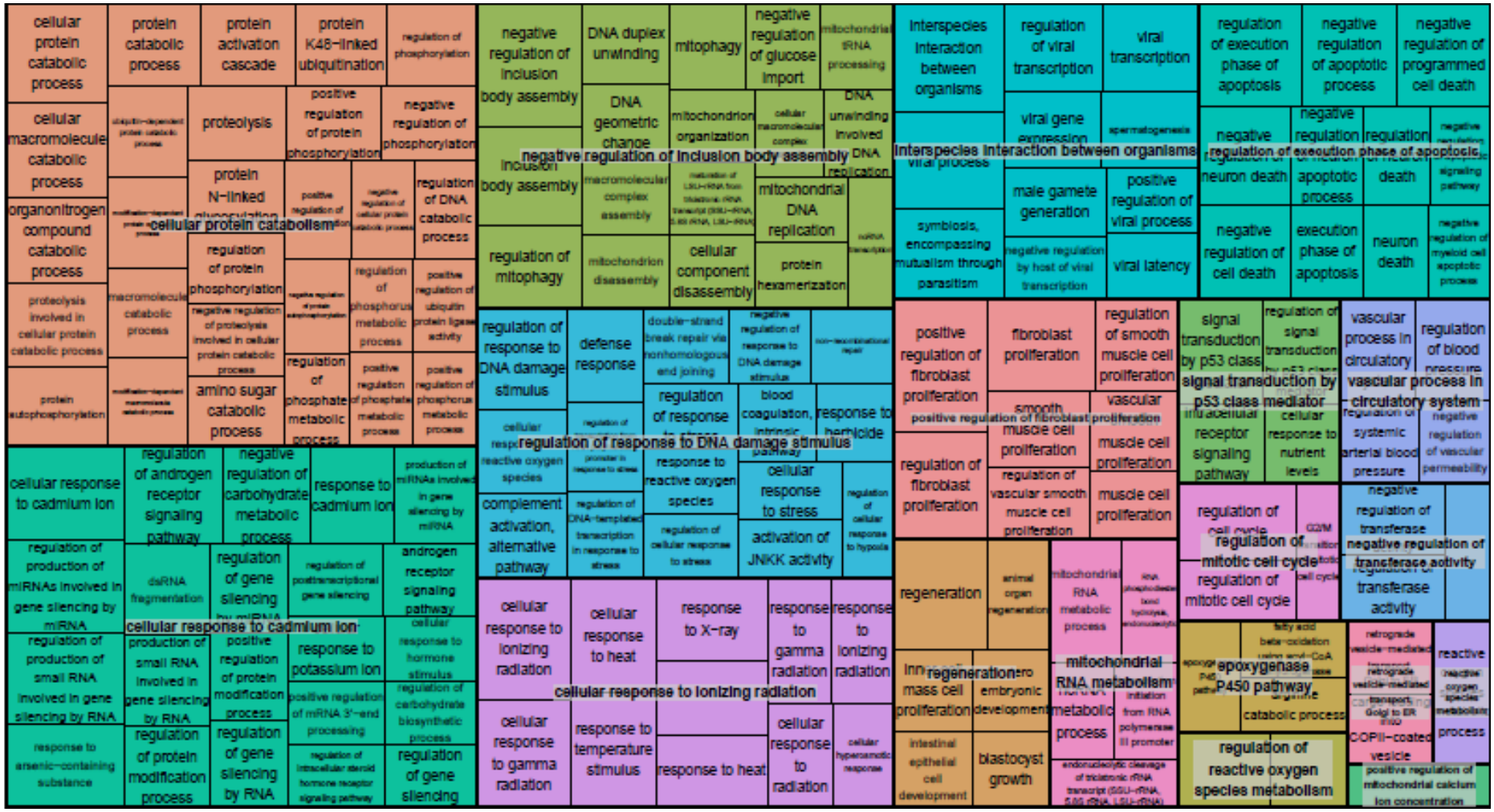


Figure 5-4A. **Pathways enriched with persistence-associated DMPs in PANC-1 (Biological Process).** REVIGO gene ontology treemap of the top 100 DMPs in infection-associated PANC-1. Pathway analysis of biological process.

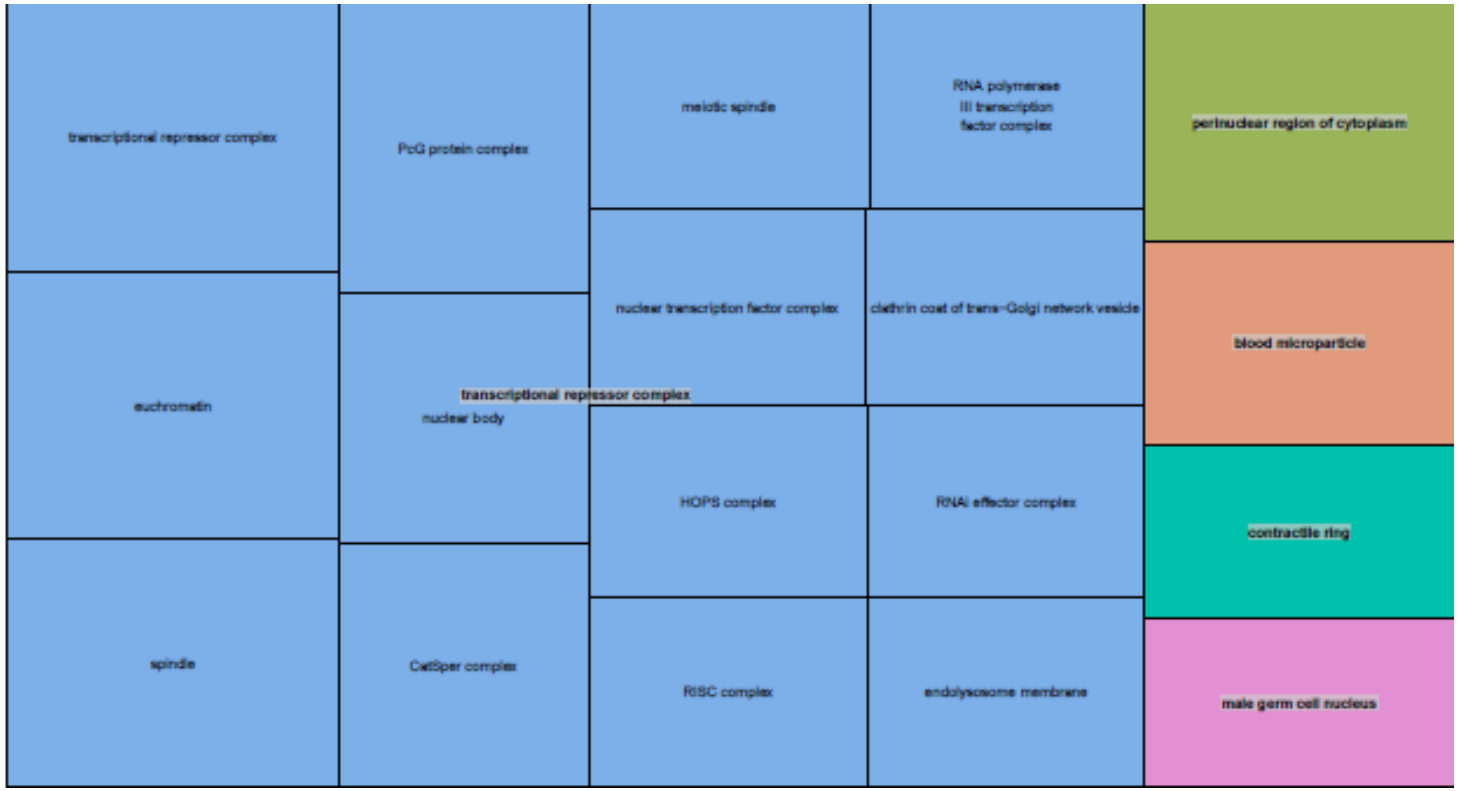


Figure 5-4B. **Pathways enriched with persistence-associated DMPs in PANC-1 (Molecular Process).** REVIGO gene ontology treemap of the top 100 DMPs in infection-associated PANC-1. Pathway analysis of *molecular process.*

5.5 Discussion

This study represents, to my knowledge, the first exploratory examination of 5mC changes associated with models of persistent Coxsackie virus infection in pancreatic cells. The cell model develops as an initial acute infection with the subsequent establishment of a persistent infection, which may resemble the situation *in vivo* during the development of persistent infection in the pancreas which leads ultimately to islet immunity. This chapter created a CBV infection model in PANC-1 cells and profiled infection-induced changes in 5mC. Length of time of infection and establishment of persistent infection were associated with DMPs in gene pathways for virus-host interactions, mitochondrial organisation, cellular protein catabolism, cell cycle regulation, DNA damage responses and cellular responses to cadmium ions.

Two persistent infections were established using different MOIs at the initial point of infection. Interestingly, both sample groups established similar characteristics of persistence in the PANC-1 cells as determined by plaque assay, which quantifies the levels of infectious virus in the supernatant. This observation is interesting because it potentially suggests that the ‘capacity’ of cells to sustain a persistent infection may be fixed within a defined range. For example, persistence may be established when the quantity of infectious virus reaches a limit whereby viral replication can proceed with a specific tolerance from the cell. Infecting cells with higher volumes of infectious virus did not lead to persistent infections characterised by higher amounts of infectious virus being released into the supernatant. Alternatively, any potential genomic changes in the virus, occurring as an adaptive response, may program the virus to replicate at a certain rate.

The infection cycle begins by attachment of Coxsackie virus to the cell surface receptor (possibly CAR) for entry into the cell, followed by transport through the cytoplasm to begin transcription and translation of the viral genome. Mature virion release from the cell usually occurs by lysis of infected cells. An acute phase was observed which was characterised by high levels of cell death as viral replication led to cell lysis and the triggering of apoptotic cellular signalling pathways. This was followed by a persistent infectious phase where PANC-1 cells displayed slightly higher cell death rates than mock-infected cells, but overall they still displayed similar behaviour to the un-infected controls.

Lower levels of cell lysis in the persistent infectious phase, as compared to the acute phase, have been observed in previous studies that established persistent models in PANC-1 cells. For example, in 2008, Kim et al. established a persistent infection with CBV3 and noted that the infection did not necessarily cause lysis. This occurred in conjunction with a viral mutation producing a form of CBV containing a 5' terminal deletion (Kim et al., 2008). Another study using the PANC-1 infection model found several mutations in the genome of CBV4 as well as changes in the cell that made it more resistant to cytopathic effects induced by CBV4 reinfection (Alidjinou et al., 2017).

A more recent study created a CBV infection model in PANC-1 using two CBV-1 strains and profiled infection-induced changes in cellular protein expression and secretion using mass spectrometry-based proteomics. Persistent infections were associated with a broad spectrum of changes including changes in mitochondrial network morphology and energy metabolism associated with the regulated secretory pathway. Interestingly, expression of cellular antiviral response proteins differed between the two persistent infections, with one strain triggering no cellular antiviral factors. This indicates that variability in infection

outcome for the host cell may be attributable to the specific virus strain. This proteomic study of infections was conducted by collecting samples of persistent infections that had been curated for a year, and running a comparison with non-infected mocks (Lietzén et al., 2019). Therefore, it was an analysis of the character of a persistence model rather than an investigation of how a persistent infection may differ molecularly to acute infections.

My study looked at changes in 5mC in the pancreatic model compared to the acute infection model to better understand the changes that occur as a result of adaptation to persistence. No DMPs reached Bonferroni level of significance but this may be due to length of time of persistence or low sample numbers (n=2). Using GO pathway analysis, the top 100 genes annotated to DMPs showed alterations in virus-host interactions, mitochondrial organisation, cellular protein catabolism, DNA damage responses and cellular responses to cadmium ions. While previous studies have demonstrated increased expression of antiviral mechanisms and proteins such as IFNL1 and EIF2AK2 (Ylipaasto et al., 2005, Richardson et al., 2009, Ylipaasto et al., 2012, Lind et al., 2013, Richardson et al., 2013, Lind et al., 2016) this study normalised for time between infections, therefore, if innate immune factors were both increased/decreased in the acute and persistent models they might not be highlighted in this study. Similarly, if secretion pathways were altered in both states of infection, this would not be highlighted as a persistence-associated change. Additionally, as mentioned not all viral strains have been associated with triggering antiviral and IFN responses.

Table 5-1 lists significant persistent infection-associated DMPs in the PANC-1 cells. From that list, G-protein coupled receptor C5 (GPRC5C) was recently found to be expressed in pancreatic islets and correlated with the expression of

genes controlling apoptosis, cell survival and proliferation. The function of GPRC5C remains to be established but it has been suggested that agents activating GPRC5C may harbour potential to restore functional β-cell mass (Amisten et al., 2017). Several of the genes listed in Table 5-1 are also involved in cell-cycle regulation, such as E3 ubiquitin-protein ligase (UHRF2), but manipulating host cell cycle is a common strategy used by viruses to generate favourable cellular environments to facilitate viral replication. For example, the capsid protein VP1 of CBV is known to induce cell-cycle arrest (Wang et al., 2019).

Pathway analysis revealed gene changes in 'interspecies interaction between organisms'. This was expected because these genes are involved in pathway defences against pathogens, as well as pathways hijacked by viruses to promote viral replication. Interestingly, 'viral latency' was generated by the pathway analysis, which could suggest that the 5mC changes associated with the persistent infection were orchestrated by the virus to benefit its survival by promoting an environment that supports viral latency.

Alterations in pathways associated with metabolic and catabolic processes were largely expected, as viruses are 'master manipulators' in redirecting cellular energy consumption to support their own genetic replication. There is limited evidence, however, on how metabolic changes in exocrine cells can influence the pathophysiology of the endocrine cells in T1D, particularly in relation to virally-induced metabolic programming. Additionally, while the potential implications of this behaviour on the development of infections in β-cells remains to be explored a number of intriguing issues arise. For example, the β-cell is a specialist glucose sensor with finely tuned processes that allow for insulin output via the sensing of small fluctuations in glucose. Certain amino

acids, including those that enhance mitochondrial metabolism such as glutamine and leucine can also stimulate insulin release and regulate the response to glucose. To control the flux of glucose, glucokinase ensures that circulating glucose concentrations are matched to metabolism, and to changes in electrical activity mediated by ATP-sensitive K^+ (K_{ATP}) channels. Ultimately, increased Ca^{2+} influx leads to insulin secretion. Virally-induced alterations to these sensitive metabolic processes could have significant effects on secretory function. In support of this, enteroviruses have been observed to manipulate voltage-gated K^+ channels in cardiac cells, and several viruses are known to regulate the activity of calcium channels. CVB protein 2B can form pores in the ER and Golgi membranes resulting in Ca^{2+} and H^+ release from these intracellular stores (de Jong et al., 2006, Campanella et al., 2004, van Kuppeveld et al., 1997). For many viruses, the point of entry provides the initial exposure to host ion channels and entry can often cause polarisation of the membranes to create a more conducive environment for infection (Charlton et al., 2020).

CBV lacks an envelope so many studies have observed that viral progeny are released from the cell via lysis of the membrane. However, they have also been observed to trigger the release of infectious extracellular microvesicles (EMVs) containing viral material. This mode of escape would allow the virus to transmit to neighbouring cells without attracting direct attention from the immune system. CBV-infected cells trigger DRP1-mediated fragmentation of mitochondria, which typically precedes mitophagy. Others have suggested that CVB can localise to the mitochondria, induce mitophagy, and subsequently leave the cell in an autophagosome-bound mitochondrion-virus complex (Sin et al., 2017). Dotta et al. (2007) noted in their analysis of human islets that virus particles could be

found in abundance in areas close to the mitochondria; many of which appeared swollen or severely damaged (Dotta et al., 2007). This supports the pathway analysis presented in this chapter which indicates that a persistent viral infection led to altered cellular mitochondrial processes. This result also agrees with the proteomics study mentioned above that observed downregulation of mitochondrial proteins, especially those involved in mitochondrial energy (Lietzén et al., 2019).

Infection-associated differential 5mC in gene pathways involved in cellular responses to cadmium ions was interesting and, perhaps, unexpected. The focus on T1D has mostly been around the immune destruction of β-cells and autoimmunity (for good reason), but the more subtle and unexpected areas can often prove interesting as well. Researchers have previously debated the significance of cadmium in the development of diabetes (Wu et al., 2017) with some evidence suggesting that chronic low level cadmium exposure impairs the function of β-cells. In MIN6 cell lines and primary mouse islets, cadmium accumulation led to inhibition of glucose stimulated insulin secretion (El Muayed et al., 2012). The relationship between cadmium ions and Coxsackie virus is also interesting with one study in mice recording an increase in intestinal absorption of cadmium during a Coxsackie virus B3 infection, which suggests the virus may have an ability alter tissue distribution of cadmium (Glynn et al., 1998). Changes in the distribution of cadmium ions in the mouse brain was also observed post-infection with Coxsackie virus, which raises some interesting questions about the virus's ability to manipulate cellular processes through ion shuttling (Ilbäck et al., 2006).

Most recently, cadmium was found to be associated with higher risk of death in patients with influenza and pneumonia, and could worsen COVID outcomes

(Park et al., 2020). Cadmium has been experimentally shown to be a potent inducer of cytokine expression *in vitro* in human fibroblasts, epithelial cells and macrophages (Låg et al., 2010), and in mouse models preexposure at a dietary intake level of cadmium was shown to potentiate pulmonary inflammation alongside infection with respiratory syncytial virus (Hu et al., 2019). These, and others studies (Krueger and Wade, 2016) suggest potential connections between cadmium ions and viral infections. However, data are limited because there have been no studies to date (to the best of my knowledge) that have investigated infection associated-differential methylation of genes responsible for cadmium ion regulation.

5.6 Caveats of this study

Persistent CBV infection models, to my knowledge, have not yet been published in an insulin producing human cell line. As mentioned in chapter two, the 1.1B4 cell line was considered as a candidate model for this study, but the analysis by Chaffey et al. (2021) has shown these to be an unsuitable choice for human studies. Previous studies have demonstrated that the 1.1B4 cell line is capable of hosting a persistent Coxsackie infection (Honkimaa et al., 2020, Nekoua et al., 2020, Chaffey et al., 2021) but these studies should be interpreted through the lens that rat cellular processes may be influencing experimental results. As such, we currently do not have a human β-cell model of persistent infection that would allow us to explore the effects of viral mechanisms on human β-cell function. However, further mechanistic and functional studies using existing PANC-1 persistent infection models would bring deeper understanding to infection-induced cellular responses. Additionally, studies are needed to map mutations in the virus acquired at the persistent phase to determine if it adapted to its host via beneficial mutations.

Had the pandemic not halted all experimental work, the intention was to take this study further and to repeat the methods with samples of persistent infection gathered beyond 70 days of the initial establishment. This study was an exploratory piece of work and to achieve efficiency , only 2 samples for each condition (i.e. mock acute, mock persistent, acute infection and persistent infection) were loaded onto the BeadChip array. This was partly due to efficiency of cost but also because we were sharing resources with the team that specialises in epigenetic profiling.

To gain more granular detail of the infection state, viral RNA could be measured using RT-qPCR to yield information about viral copy number and also fixed cells would be prepared for FACS staining to assess viral dsRNA levels. IHC would also be conducted to confirm the presence of VP1 protein in the cell. The impact of infection on innate immune and cell survival pathways in the infected and uninfected cells can be assessed by Qiagen Type 1 interferon and antiviral response RT2 Profiler PCR Arrays, multi-parameter flow cytometry and IF analsysis. Cytokine and chemokine increase can be asssessed using either sandwhich ELISA or a BD Flex Set Cytokine Bead Array.

No single cell line is superior for studying all enteroviral types, therefore, the results from this study may not represent the viral behaviour of other Coxsackie serotypes. However, this model can be used to test the ability of other relevant CBV serotypes (identified through population studies and ongoing RT-PCR studies in nPOD cases) to produce persistent infections.

Chapter 6

6 General Discussion and Conclusions

The overarching aim of this thesis was to determine if immunomodulatory proteins such as PD-L1 were involved in the development of T1D. The results presented have concluded that immunomodulatory proteins are indeed present within islets from individuals with T1D. Based on the known functions of these proteins, they most likely regulate autoimmune T-cells responses in T1D. A secondary aim of this thesis was to investigate additional methods for 'crosstalk' between β-cells and immune cells by developing effective methods for isolation of EVs from β-cell lines and to characterise expression of PD-L1 on the vesicle surface. The results demonstrated that PD-L1 expression is present on both microvesicles and exosomes. The additional aims and project plans for this thesis were initially to investigate how persistent enterovirus infection can alter the β-cell environment and utilise the EV transport system to infect neighbouring cells and, potentially, alter the crosstalk between β-cells and immune cells. However, the COVID-19 pandemic resulted in a national lockdown and closure of laboratories. Whilst away from the laboratory, I undertook a project using freely available public data where I could investigate the occurrence of PDAC with diabetes. This formed the third aim of this thesis and allowed me to improve my knowledge on pathological conditions of the pancreas. In particular, it provided an opportunity to focus on the exocrine tissue since the majority of research in T1D centres on the β-cell. PDAC associated T3cDM is not a well understood area of diabetes and is largely overlooked in the literature because research has focused on the biology of the cancer. The TCGA database is a widely used resource and has been utilised in hundreds of studies across 33 cancer types, but I recognised that researchers had not analysed the RNA expression profiles for PDAC samples based on clinical variables such as diabetes. Therefore, the third aim of this thesis enabled me to improve my

knowledge of the pancreas, develop new skills in bioinformatics, and analyse an understudied area of diabetes, as well as reveal information of relevance to clinical management.

T1D is an autoimmune disease caused by immune-mediated β-cell dysfunction and apoptosis, leading to the lifelong requirement for exogenous insulin therapy. There is a complex dialogue between invasive autoreactive immune cells and β-cells within the islets of Langerhans. The immune cells release chemokines and cytokines into the islet microenvironment and deliver intercellular pro-apoptotic signals, while the β-cells can generate antigenic signals from degraded products such as insulin or stressed (or dying) β-cells which attract and activate the immune cells (Eizirik et al., 2009, Gonzalez-Duque et al., 2018, Thomaidou et al., 2018). This complex dialogue is influenced by genetic factors, age and environmental factors such as viral infection and diet (DiMeglio et al., 2018, Op de Beeck and Eizirik, 2016, Ilonen et al., 2019). The immune attack results in local inflammation (insulitis) and progressive β-cell dysfunction and death, mainly via apoptosis (Eizirik et al., 2009, Todd, 2010, DiMeglio et al., 2018). There exists no cure or therapeutic approaches to prevent T1D, although a monoclonal antibody against CD3 is currently being trialled to delay the onset of disease (Herold et al., 2019). Severe β-cell dysfunction is suggested to precede β-cell death as patient donor samples have shown 40-50% normal β-cell mass before death from ketoacidosis (Gotthardt et al., 2014, Oram et al., 2019). Islets affected by severe insulitis in NOD mice are dysfunctional when isolated, but regain function once removed from the infiltrating immune cells after several days in culture (Strandell et al., 1990). A similar observation has been made in human

islets transported from T1D donors into a nondiabetogenic environment *in vitro* (Krogvold et al., 2015b).

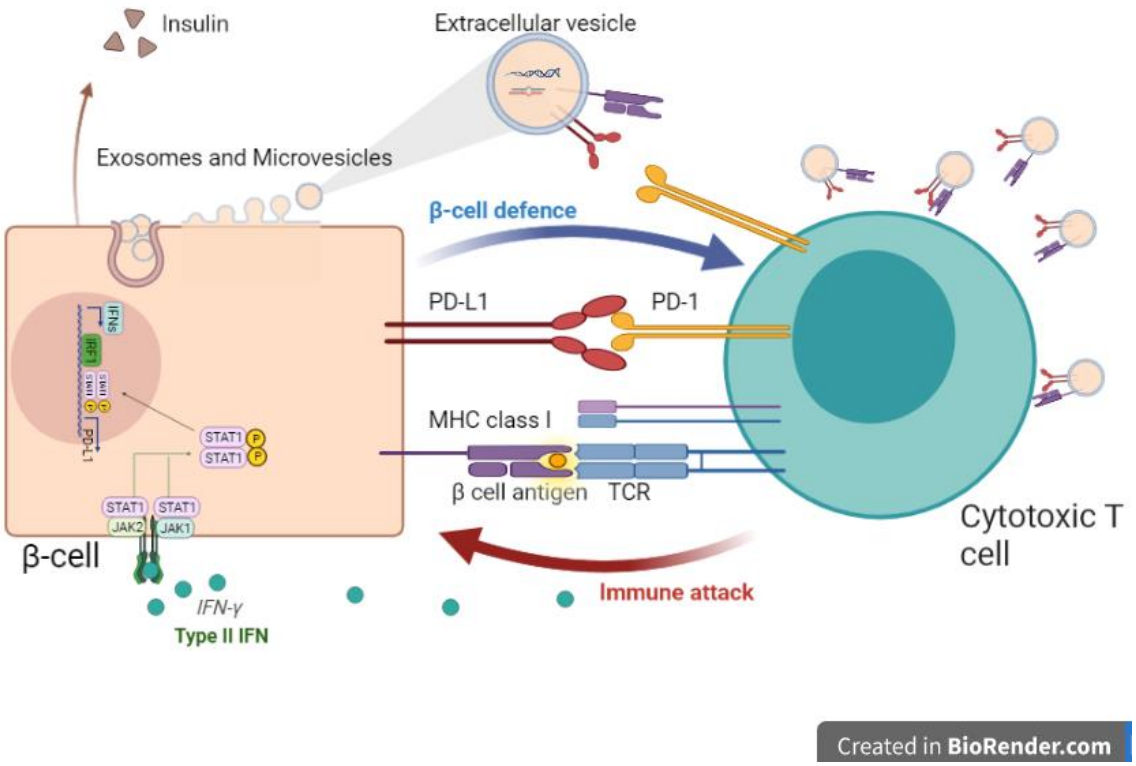


Figure 6-1 The PD-L1 defence mechanism employed by β-cells to delay the immune attack. PD-L1 is regulated by IFNs and the JAK/STAT/IRF1 pathway. PD-L1 binds its receptor binding partner PD-1 on the surface of cytotoxic T cells to negatively regulate T cell function. Extracellular vesicles carrying PD-L1 may enhance the β-cell defence against immune attack by creating a cloud of PD-L1 to bind PD-1.

This thesis explored the dialogue between islets and the immune system, particularly the cellular defences deployed by endocrine cells to delay the immune assault. **Chapter two** investigated the expression of PD-L1 within insulin-containing islets and found PD-L1 to be expressed on the β-cells of individuals with T1D, but not in individuals without T1D or insulin-deficient islets. PD-L1 expression was observed in the islets of individuals with T1D diagnosed at varying ages and it correlated with expression of IRF1 and the level of immune infiltration within the islet. This indicates that PD-L1 is a marker of IFN-induced inflammation within the islet. As mentioned, not all islets within the pancreas show evidence of insulitis, and expression of PD-L1 followed this observation as not all β-cells expressed PD-L1. Collaborative studies with our colleagues at the University of Brussels demonstrated that expression of PD-L1 in EndoC-βH1 cells is regulated by IFNs via the JAK/STAT and IRF1 pathways.

Type 1 IFNs are known to be present in the islets of individuals with T1D during the early stages of inflammation, while IFN-γ together with IL-1β, tumour necrosis factor (TNF) and, potentially, IL-17 will trigger ER stress and upregulation of HLA class I at the late stages of disease (Morgan and Richardson, 2018, Marroqui et al., 2017). These events were previously shown to lead to cell death and the augmented presentation of neoantigens to immune cells (Eizirik et al., 2009, Gonzalez-Duque et al., 2018). However, **Chapter two** revealed that not all of these cytokines are deleterious to β-cells and that both IFN-α and IFN-γ upregulate the expression of PD-L1 and HLA-E on β-cells, although HLA-E is predominantly found on the α-cells. These proteins provide a negative feedback to T cells and natural killer cells and, at least in animals

models, protect β-cells against immune-mediated cell death (Martinov and Fife, 2020).

PD-L1 and its receptor binding partner PD-1 probably play a protective role in individuals with T1D, as their blockade in the context of cancer therapy is associated with development of T1D, amongst other endocrine disease. Most downstream proinflammatory and proapoptotic signals delivered by IFNs to β-cells are mediated by the transcription factors STAT1 and STAT2 (Moore et al., 2011, Colli et al., 2018) but PD-L1 is also regulated through the IFN/STAT/IRF1 pathway (Figure 6-1). The observation that IFNs are observed in islets isolated from individuals with T1D within the first few weeks to months from point of diagnosis (Lundberg et al., 2016) indicates that PD-L1 could be expressed in the earlier stages of disease. Therapeutic approaches should take note of the PD-L1/PD-1 axis and approaches should involve preserving signals downstream of IFNs that would promote PD-L1 expression, whilst blocking deleterious signals such as classical HLA class I expression, ER stress, and chemokine production (Trivedi et al., 2017).

Other research teams have taken note of our published work on PD-L1 (Colli et al., 2018), and below is a short summary of selected studies that have investigated PD-L1/PD-1 further in the context of diabetes.

i) Yoshihara et al. (2021) generated human islet-like organoids from induced pluripotent stem cells and showed that overexpression of PD-L1 protected the organoid xenografts such that they were able to restore glucose homeostasis in immune-competent diabetic mice for 50 days. Furthermore, *ex vivo* stimulation with IFN-γ induced

endogenous PD-L1 expression and restricted T cell activation and graft rejection. This is significant because glucose-responsive islet-like organoids that are able to avoid immune destruction provide an alternative to current therapies (ex. cadaveric) in the treatment of T1D (Yoshihara et al., 2020).

ii) Li et al. (2020) found that PD-1 on CD4+/CD8+ T cells showed a dynamic upregulation around the partial remission phase in T1D. The partial remission phase was associated with restoration of PD-1/PD-L1 on CD4+ and CD8+ T cells, suggesting that PD-1/PD-L1 may be a involved in prolonging this phase (Li et al., 2020b)

iii) Ma et al. (2020) treated β-like cells with activated T cell-conditioned medium or co-cultured β-cells with cognate T cells and observed an upregulation in PD-L1 expression. PD-L1 overexpression did not interfere with human β-like cell differentiation and function and the authors speculated it likely had therapeutic effects by reducing β-cell targeted autoimmunity (Ma et al., 2020).

iv) Castro-Gutierrez et al. (2021) presented an *in vitro* assay to measure autologous CD8 T cell stimulation against stem cell-derived β-like cells (sBC) in a human setting. Upon pro-inflammatory cytokine exposure sBC upregulate HLA class I molecules (which allows for recognition by CD8+ T cells). To protect sBC's from immune assault, genome engineering was used to delete surface expression of HLA class I molecules and they integrated an inducible overexpression system for PD-L1 expression. These engineered sBC with overexpression of PD-L1 showed reduced stimulation of diabetogenic CD8 T cells when compared to unmodified cells. This provided

protection from diabetes-specific immune recognition in a human setting (Castro-Gutierrez et al., 2021).

v) Shan et al. (2021) found that decreased expression of PD-1 on CD8+ effector memory T cells correlated with the pathogenesis of T1D (Shan et al., 2021).

Further studies are needed to fully understand the relationship between PD-1 on CD8+ T cells and PD-L1 on β-cells in the context of T1D, and it may be that polymorphisms exist within the T1D population that render the PD-1/PD-L1 pathway less effective, potentially contributing to the heterogeneity we see in disease pathology across individuals with diabetes.

The expression of PD-L1 was explored further in **Chapter three** with the analysis of EV release from EndoC-βH1 cells. Exosomes and microvesicles were successfully isolated from PANC-1 and EndoC-βH1 cells and contained the typical exosome and microvesicle markers as seen in EVs isolated from other organs. EVs differed in polydispersity when collected at different centrifugal forces, with larger vesicle populations collected at 10,000 x g. EVs released from PANC-1 and EndoC-βH1 cells treated with high and low glucose were isolated and demonstrated differing population characteristics. EVs isolated from EndoC-βH1 cells had less polydispersity, potentially indicating these cells have cellular mechanisms that act to closely regulate vesicle release during varying extracellular conditions, and these may act similar to regulatory processes involved in glucose-stimulated insulin vesicle release. PD-L1 was found to be expressed on the surface of EVs isolated from PANC-1 and EndoC-βH1 cells and was significantly upregulated by IFNs. EndoC-βH1 cells were

found to have substantially higher levels of PD-L1 than PANC-1 cells, which was expected following the results from **Chapter two**. This is important because PD-L1 on EVs may enhance the defence against autoreactive T cells. Indeed, PD-L1 on exosomes have previously been observed to prevent immune destruction of tumour cells (Chen et al., 2018) so this process may also be relevant in T1D. The β-cells could potentially be releasing a 'cloud' of EVs containing PD-L1 to deter the immune attack (Figure 6-1).

Future experiments would involve isolating EVs from the islets of individuals with T1D to determine if they express PD-L1 on the vesicle surface. If specific markers of β-cells could be identified on EVs, then EVs collected in blood samples from individuals with T1D could be used as biomarkers to indicate levels of inflammation and immune infiltration into the islets. EVs have already been identified and developed as biomarkers of cancer to provide clinicians with early warning signs of disease. As EV detection technology develops, there is the potential to translate this field into pre-diabetes diagnostics.

Chapter four undertook an analysis of patients with PDAC and diabetes (T3cDM). The onset of diabetes in later life is being investigated by others as a potential early marker of PDAC development, as PDAC is believed to be the cause of the diabetes in a subset of individuals. PDAC currently has a poor 5-year survival rate and is more closely associated with diabetes than any other type of cancer. A poor 5-year survival rate results, in part, from a lack of effective immunotherapy options. Unlike many other cancers, PDAC does not respond well to anti-PD-1 or anti-PD-L1 immunotherapy.

Previous studies (Bailey et al., 2016) have categorised PDAC into four subtypes based on RNA expression profiles of key markers. Many of those markers are

associated with pancreatic endocrine cell development and differentiation (ex. *INS, MAFA, PAX4, PDX1*). Others, such as the markers defining the immunogenic subtype, are immune checkpoint markers such as *PDCD1 (PD-1)* and *CTLA4*. Despite a substantial number of PDAC patients experiencing concurrent diabetes, the categorisation of subtypes did not take into account RNA expression signatures that could be associated with clinical variables such as diabetes. Additionally, past studies looking at diabetes in individuals with PDAC combined patients who had a history of chronic pancreatitis with those who had not. Chronic pancreatitis is a known risk factor for diabetes and PDAC, and many germline mutations associated with chronic pancreatitis are also associated with PDAC. Therefore, **Chapter four** investigated whether any of the tumour subtypes were derived from individuals with diabetes but without a history of pancreatitis and analysed the gene expression differences between individuals with PDAC and diabetes vs. those with PDAC and no diabetes. Contrary to previous studies (Yan et al., 2017), I found no indication that the diabetes cohort were derived from the immunogenic subtype and there was no significant upregulation in *PDCD1*and *CTLA4* within that cohort. This indicates that individuals with diabetes and PDAC are not immunosuppressed and should not be treated separately to other PDAC patients, as recommended by others (Yan et al., 2017). Gene expression analysis also revealed that expression of *INS, GCG,* and *SST* were all significantly reduced in the diabetes cohort, potentially suggesting that T3cDM does not follow the typical characteristics of T2D, where *GCG* and *SST* would not be significantly reduced. Additionally, study of tissue sample sections revealed that islets from individuals with PDAC, particularly within the neoplasm, have thickened basement membranes. The

significance of this is unknown and work could be undertaken to understand the mechanisms directing basement membrane thickness.

Finally, **Chapter 5** investigated the epigenetic changes within a host cell (PANC-1) that occurred as a result of persistent infection with CBV. This in important because CBV is a known environmental trigger for T1D in young children, and there is a lack of research on how persistent infections can alter the pancreatic environment. This investigation went beyond previous studies as it focused on cellular changes that occurred as a result of the PANC-1 cells transitioning from hosting an acute infection to a persistent infection. No changes in gene methylation profiles were observed to reach statistical significance, but this may have been due to the low sample number or length of time of infection used experimentally. Elongating the length of time of infection to several months may result in significant methylation events occurring in the host cell. However, changes were observed in CpG sites associated with viral latency, mitochondrial function and metabolism, ion channels, and cadmium channels. These were largely expected as past studies on CBV infection have demonstrated that CBV can manipulate the host cell to redirect energy metabolism and modulate mitochondrial function. Other virus types (ex. Human cytomegalovirus, influenza) are also known to modulate ion channel activity to a create a more conducive environment for viral replication and evasion of the immune system (Mankouri et al., 2009, Londino et al., 2017, Charlton et al., 2020). CBV may well be using these processes (evolutionary strategies) for similar purposes, but modulation of host metabolism, mitochondrial function, ion channel activity and processes promoting viral latency would all support a persistent state of infection within PANC-1 cells. CBV is likely altering gene expression in the host cells to support low levels of viral replication and prevent

cell death. Future studies need to look at models of persistent infections in β-like cells to observe how viral behaviour can affect insulin release mechanisms, mitochondrial function, ion channel function, cellular apoptosis, and host anti-viral defence mechanisms.

In 2019, I was given the opportunity to work at the Cabinet Office, Whitehall, on a 4-month secondment. The goal of the secondment was to learn how to translate academic research into evidence for policy development in Government. Research is often presented to a specialist audience, resulting in technological advancements or best practice approaches being excluded from the policy making process because they are inaccessible, either physically because of journal paywalls or because they require specialist knowledge. There is also a lack of training across the whole of academia in translating scientific advances into formats that can inform policy. This is important because it means that industry and pharmaceutical companies will often have more influence than academia in policy decisions that affect the health and wellbeing of UK citizens. It also affects the funding made available for research and innovation across the UK because academics are not able to present their research and develop convincing reasoning behind the need for additional resources.

The training and networks I built from working at the Cabinet Office led to my appointment in NHS Test and Trace. From May to September 2020, I assisted the Chief Medical Advisor, Dr. Paul Cosford followed by Dr. Susan Hopkins (Public Health England), in delivering clinical advice across the Test and Trace programme. One of my tasks was to translate the scientific evidence published by SAGE and Imperial College London to inform decisions made at the

executive level of the Joint Biosecurity Centre, Public Health England, Cabinet Office and Department of Health and Social Care. I attended weekly meetings with Ministers and the Chief Medical Officer, Prof. Chris Whitty, to ensure that published Government guidance had appropriate clinical oversight and was evidence-based. I also highlighted the need for Test and Trace to acknowledge that people with diabetes are at high risk of death from COVID-19, and therefore need additional support.

The provision of healthcare services across the UK has changed significantly over the past fifteen years, and governance/oversight has largely been transferred away from local services. As a result, public health decisions affecting the whole of the UK are largely made at a centralised level, and based in London, UK. The main challenge I faced while working in Test and Trace was ensuring adequate support for local authorities and local health services to control the spread of COVID-19. As the pandemic eases, there will be much opportunity for reflection, but the events of 2020-21 have demonstrated that the UK's most vulnerable citizens, including individuals living with diabetes, are at risk if they do not receive adequate support at a local and regional level

Publications arising from this thesis

(*) denotes co-first authorship

Colli, Maikel L.*, **Jessica L. E.*** Hill, Laura Marroquí, Jessica Chaffey, Reinaldo S. Dos Santos, Pia Leete, Alexandra Coomans de Brachène, Flavia M. M. Paula, Anne Op de Beeck, Angela Castela, Lorella Marselli, Lars Krogvold, Knut Dahl-Jorgensen, Piero Marchetti, Noel G. Morgan, Sarah J. Richardson, and Décio L. Eizirik. 2018a. 'PDL1 is expressed in the islets of people with type 1 diabetes and is up-regulated by interferons IFN-α; and-IFN-γ; via IRF1 induction', *EBioMedicine*, 36: 367-75.

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