Abstracts by Title

Viral Etiology 4

Detection of Enteroviruses in Tissue Samples of Cadaver Organ Donors with Type 1 Diabetes 4

Detection of Enteroviral Proteins and Cellular Antiviral Responses in the Islets of Type 1 Diabetes Patients – A Comparative Analysis of Two Cohorts 5

Different Species of Enteroviruses (EV) in Peripheral Blood Leukocytes (PBL) of Children at the Clinical Onset of Type 1 Diabetes 6

MHC Class I on Pancreatic Islets from Longstanding Diabetes Patients: Persistent Hyperexpression is Restricted to Type 1 Diabetes and Does Not Correlate with Enteroviral Infection, Infiltration or Insulin Depletion 7

Examination of Human Pancreatic Tissue and Isolated Pancreatic Islets from Organ Donors with Diabetes-associated Autoantibodies 8

Autoantigens, T Cells and TCRs: Human Disease-specific Therapeutic Targets 9

In-situ Detection of Islet Antigen-specific CD8 T Cells in Insulitic Lesions of New-onset and Long-term Type 1 Diabetes Patients 9

Identification of GAD65 and Proinsulin-specific CD4+ and CD8+ T Cells in Pancreatic Lymph Nodes, Spleen, and PBMC from Subjects with T1D and Islet Autoantibody Positivity 10

454 Pyro-sequencing of T Cell Receptors Targeting Pancreatic Islets 11

Monoclonal Expansions of TCR in a Diabetic Pancreas 14

Autoreactive B Cells from Spleen and Pancreatic Draining Lymph Nodes from Type 1 Diabetes Subjects 15

Enhancement of Treg-mediated Supression by NKT Cells in T1D 16

Heterogeneity of Pancreas Pathology and Disease Mechanisms 17

Expression and Regulation of Chemokines in Human Islets and Type I Diabetes 17

Loss of Beta Cell Heparan Sulfate and Expression of Heparanase by Insulitis Mononuclear Cells Correlates with Type 1 Diabetes in Human Pancreas Specimens 18

Specific Extracellular Matrix Components Accumulate in Islets and Lymphoid Tissues of Type 1 Diabetic Patients 19

Down Regulation of Glucokinase and Mitochondrial ATP Synthase in Islets from Type 1 Organ Donors 20

Complement Activation in Type 1 Diabetes: Analysis of Pancreatic Tissue from nPOD Cases 21

Adhesion Molecules on High Endothelial Venules of Pancreatic Lymph Nodes from Humans with Type 1 Diabetes 22

Revelation of Altered Expression of B7-H4: Decrease in Type 1 Diabetes and Increase in Insulinoma 23

From Genetics to Gene Expression in nPOD Tissues 24

Disease-dependent and Tissue-specific Changes in Gene Expression in Type 1 Diabetes (T1D) 24

Identification of Extra-thymic Aire-expressing Cells (eTACs) in Human Tissue and Comparison between Type-1 Diabetic and Non-diabetic Controls 25

ImmunoChip Data on nPOD Samples 26

Characterizing Gene Expression in nPOD Donor Islets 27

Peripheral Blood Monocyte Gene Expression Profile and Pancreatic Monocyte Infiltration in Patients with Type 1 Diabetes 28

Whole Genome Scale DNA Methylation Differences in Type 1 Diabetes Disease-related Cells 29

Emerging Technologies and Novel Research Avenues **30**

Feasibility of Flow Cytometric Analysis of nPOD Bone Marrow Specimens 30

Flow Cytometric Immunophenotyping of nPOD Donors 32

Development of T1D in Humanized Mice Engrafted with Tissues from Autoimmune Donors 33

Quantum Dot MHC Multimers 34

The Role of IA-2 Extracellular Domain in Type 1 Diabetes Progression 35

In-situ Reduction Coupled with Imaging Mass Spectrometry as a Novel Method for T1D Biomarker Identification in nPOD Tissue Samples 36

In Vitro Evaluation of Non-Specific Binding of the Candidate Beta Cell Mass PET Probe (+) 18F-FP-DTBZ 37

Pancreas and Beta Cell Biology **38**

The Pancreatic Duct Gland Compartment in Non-Diabetic and Type 1 Diabetic 38

Subjects 38

Formation of a Baseline Pancreatic β-cell Mass in Humans is Completed Early in Life 39

β-cells Persist in Some T1DM Pancreata without Attempted Evidence of β-cell Turnover nor Insulin-Glucagon Co-expression 40

Cytoplasmic-Nuclear Trafficking of G1/S Cell Cycle Molecules: A Novel and Critical Regulatory Mechanism Controlling Adult Human Beta Cell Replication 41

Residual Beta Cells in Autoimmune Type 1 Diabetes Mellitus 42

Case Processing **43**

nPOD Case Processing 43

Abstracts by Name (either senior author, presenter, or nPOD PI)

Anderson, Mark
Atkinson, Mark
Beyan, Huriya
Bogdani, Marika
Brusko, Todd
Butler, Peter
Campbell-Thompson, Martha
Cheung, Susan
Coppieters, Ken (MHC)
Coppieters, Ken (*In Situ*)
Fathman, Garry
Fiaschi-Taesch, Nathalie
Frisk, Gun
Gerling, Ivan
Gianani, Roberto
Green-Mitchell, Shamina
Greiner, Dale
Harris, Paul
Homann, Dirk
Hyöty, Heikki
Jacobson, Dan
Kent, Sally
Kushner, Jake
Leslie, David
Lightfoot, Yaíma
Madeddu, Paolo
Martè, Mercí
Mathews, Clayton
McMahon, Eileen
Michie, Sara
Montgomery, Emily
Morgan, Noel
Morran, Michael
Nadler, Jerry
Nakayama, Maki
Onengut-Gumuscu, Suna
Pietropaolo, Massimo
Reijonen, Helena
Rhodes, Chris
Rich, Stephen
Richardson, Sarah
Roep, Bart
Roura-Mir, Carme
Rowe, Patrick
Sarkar, Suparna
Simeonovic, Charmaine
Stewart, Andy
Thomas, Ranjeny
Toniolo, Antonio
von Herrath, Matthias (MHC)
von Herrath, Matthias (*In Situ*)
Warnock, Garth
Wight, Thomas

Viral Etiology

Detection of Enteroviruses in Tissue Samples of Cadaver Organ Donors with Type 1 Diabetes

Maarit Oikarinen, Sisko Tauriainen, Sami Oikarinen, Teppo Haapaniemi, Teemu Honkanen, and Heikki Hyöty
Department of Virology, School of Medicine, University of Tampere, Tampere, Finland

Purpose: Epidemiological studies have showed an association between enterovirus (EV) infections and type 1 diabetes (T1D). Prospective studies have suggested that EVs may initiate the beta-cell damaging process. Potential causal relationship has got support from studies showing enterovirus in the pancreatic islets and intestinal mucosa of T1D patients. The aim of the present study was to analyze the presence of enterovirus in the pancreas, spleen, duodenum and pancreatic lymph nodes (PLN) of T1D patients, islet-autoantibody-positive (auto-ab+) individuals and healthy controls.

Methods: Tissues were collected from cadaver organ donors in the Network for Pancreatic Organ donors with Diabetes (nPOD) study. Formalin-fixed paraffin-embedded tissue samples were analyzed using enterovirus-specific *in situ* hybridization (ISH) and immunohistochemical (IHC, clone 5-D8/1, DakoCytomation) assays. Study series included 18 T1D patients from whom 21 pancreas sections (two sections from one patient and three from another patient taken from different parts of the pancreas), 15 spleen, 10 duodenum and 2 PLN sections were available. Similar samples were available from 3 auto-ab+ individuals including 4 pancreas sections (2 from same individual), 3 spleen, 2 duodenum and 1 PLN sections. Samples from 23 non-diabetic controls included 25 pancreas sections (two sections were available from two individuals), 17 spleen and 1 duodenum sections.

Summary of Results: Altogether 43 % of all samples of T1D patients and 40 % of all samples of auto-ab+ individuals compared to only 9 % of all samples of control donors were positive for EV genome in ISH. Virus protein was found by IHC in 29 % of T1D patients, 38 % for auto-ab+ individuals and 5 % of healthy controls. EV was detected more frequently in the pancreas (30 % vs. 4 %; p=0,017) and spleen (53 % vs. 18 %; p=0,034) of T1D patients than control subjects using ISH. IHC showed enterovirus in 33 % of the pancreas samples of T1D patients but in only 5 % of control subjects (p=0,015). One T1D patient was positive in only one part of the pancreas in ISH and one in IHC. Virus isolation was carried out from 9 pancreas and 11 spleen samples, and EV RT-PCR from 8 pancreas, 10 spleen and 2 duodenum samples, but none were positive.

Conclusions: EV was detected more frequently in the pancreas and spleen of T1D patients than in control subjects using ISH and IHC. The results support the role of enteroviruses in the pathogenesis of T1D and fit with persisting slowly replication infection.

Detection of Enteroviral Proteins and Cellular Antiviral Responses in the Islets of Type 1 Diabetes Patients – A Comparative Analysis of Two Cohorts

Sarah Richardson[1], Shalinee Dhayal[1], Adrian J. Bone[2], Alan K. Foulis[3], and Noel G. Morgan[1]
[1]Endocrine Pharmacology, Peninsula College of Medicine and Dentistry, Plymouth, UK; [2]Cell and Molecular Biology, School of Pharmacy and Biomolecular Sciences, Brighton, UK; [3]Royal Infirmary, Glasgow, UK

Purpose: nPOD type 1 diabetes cases with disease duration of less than 8 years were examined to establish whether islet endocrine cells display evidence of enteroviral infection. nPOD non-diabetic controls, autoantibody positive cases and patients diagnosed with type 2 diabetes were also studied, and a comparison made with a previously analysed UK cohort of diabetic patients.

Methods: Formalin-fixed paraffin embedded pancreatic sections from selected nPOD cases (6 type 1 diabetes; 7 type 2 diabetes, 10 non-diabetic controls and 3 young (<24y) autoantibody positive), were examined by immunohistochemistry for the presence of the enteroviral capsid protein vp1 (Dako antibody; 5D8/1), insulin, protein kinase R (PKR) and class I MHC. Results were compared with those of a previously analysed UK cohort, consisting of 72 cases of recent-onset type 1 diabetes and 50 non-diabetic paediatric and neonatal controls, as well as 25 adult patients with type 2 diabetes and 69 non-diabetic adults (Richardson et al, Diabetologia 52; 1143-51, 2009).

Summary of Results: Multiple intensely vp-1 positive islet cells were observed in many insulin-containing islets (ICI) of 4 of 6 (67%) nPOD type 1 diabetes cases. Of the two cases having no evidence of vp1 expression, one was devoid of ICI. Thus, vp1 was present in 4 of 5 cases with ICI (80%). Only 2/10 (20%) non-diabetic controls had vp1 positively stained islet cells and these were also only rarely detected in the islets of autoantibody positive cases. One striking difference between the type 1 diabetes cases from the nPOD and UK cohorts was a dramatic increase in the number of individual endocrine cells within any given islet which were immunopositive for enteroviral vp1 in the nPOD cases. The expression of vp1 correlated with an increase in protein kinase R expression within the islets of the nPOD cases studied - a finding that was confirmed in the UK cohort, where vp1 and PKR co-localised within individual beta-cells. These results imply that enteroviral infection occurs commonly in type 1 diabetes and that an anti-viral response is mounted in infected islet cells. Analysis of vp1 staining in the nPOD type 2 diabetes cohort (predominantly young-onset; 18-44y, with one older case; 76y) yielded unexpected results: 5 of 7 cases (all young) demonstrated multiple intensely positive vp1 cells within the islets, and hyperexpression of class I MHC antigens by islet endocrine cells was detected in the islets of 4 of these cases. These findings were not reproduced in the UK type 2 diabetes cohort (all older patients), where only occasional immunoreactive vp1 positive endocrine cells were found in 40% of cases, and MHC I hyperexpression was never seen.

Conclusions: Enteroviral vp1 expression is observed at high frequency in the ICI of type 1 diabetes cases in both the nPOD collection and in a separate (older) UK cohort. In both cohorts, enteroviral vp1 expression correlated with increased expression of the pathogen-recognition receptor, PKR and with hyperexpression of class I MHC. The number of individual islet cells that were immunopositive for vp1 was much higher in the nPOD than in the UK cohort. Among the nPOD samples, the islets of patients diagnosed with type 2 diabetes at an early age, also displayed evidence of marked vp1 expression. This was associated with hyperexpression of class I MHC, which was never seen among type 2 diabetes cases in the UK cohort, raising the possibility that some of the nPOD patients do not have typical type 2 diabetes.

Different Species of Enteroviruses (EV) in Peripheral Blood Leukocytes (PBL) of Children at the Clinical Onset of Type 1 Diabetes

Antonio Toniolo[1], Andreina Baj[1], Martina Colombo[1], and Alessandro Salvatoni[2]
[1]Department of Clinical and Experimental Medicine, Laboratory of Microbiology, University of Insubria Medical School, Varese, Italy; [2]Department of Clinical and Experimental Medicine, Pediatric Endocrinology Unit, University of Insubria Medical School, Varese, Italy

Purpose: EV have long been suspected to trigger T1D. To verify this hypothesis, EV infectivity and genome were searched for in PBL of pediatric patients on the day of clinical diagnosis.

Methods: 112 children developing diabetes at two Pediatric Endocrinology Centers of Northern and Central Italy (median age 9.5 yrs; range 2-16 yrs) were studied. EV-susceptible cell lines (RD, HeLa, AV3, CaCo) were immediately co-cultured with the patients` PBLs. Primers covering the 5`UTR, VP4, and 3D genome regions of 100 EV types were used in highly sensitive RT-PCR assays that were run both on plasma and tissue culture medium from cell lines exposed to patients` PBLs. Expression of viral capsid proteins was evaluated in "infected" cell cultures with mAbs directed to the capsid protein VP1. Immunoassays were used to quantify cytokines released by cultured cells. Routine methods were used to measure levels of blood glucose, HbA1c, C-peptide (time 0 and 6 min after glucagon stimulation), diabetes related auto-Abs (GAD65, IA2, ZnT8, IAA), and - one year after diagnosis - the insulin requirement (IU/Kg/day).

Summary of Results: EV infectivity and genome fragments were found in PBLs of 89/112 (79%) children, versus 2/69 (2.8%) matched non-diabetic controls. EV of the B species were predominant (58% of positives). Viruses of the A, C, and D species were also detected. Tests on infected cell lines confirmed the intracellular production of viral capsid proteins and of the MCP1 chemokine. As compared to EV-negative children, EV-positive patients had significantly reduced levels of glucagon-stimulated C-peptide and significantly higher levels of HbA1c at diagnosis. However, titers of diabetes-related auto-Abs were not different and - one year after diagnosis - the insulin requirements of the two groups were comparable.

Conclusions: The presence of EV in blood is a frequent and significant biomarker of early stage T1D. Collaborative studies are needed to identify the EV types associated with T1D in different geographic areas. We thank: CARIPLO Foundation (IT), VIDIS Group (UK), Gianni Valcavi, Attorney.

MHC Class I on Pancreatic Islets from Longstanding Diabetes Patients: Persistent Hyperexpression is Restricted to Type 1 Diabetes and Does Not Correlate with Enteroviral Infection, Infiltration or Insulin Depletion

Ken T. Coppieters[1], Anna Wiberg[1], Natalie Amirian[1], Sally Kent[2], Thomas W. H. Kay[3], Peter D. Campbell[3], Mark Atkinson[4], Heikki Hyöty[5], Steven Tracy[6], and Matthias von Herrath[1]

[1]Type 1 Diabetes Center, La Jolla Institute for Allergy and Immunology, La Jolla, CA; [2]Division of Diabetes, Department of Medicine, University of Massachusetts Medical School, Worcester, MA; [3]St Vincent's Institute, Fitzroy, Victoria, Australia; [4]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL; [5]Department of Virology, School of Medicine, University of Tampere, Tampere, Finland; [6]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE

Purpose: Major Histocompatibility Class (MHC) class I antigens present intracellular peptides to CD8 T cells and render the expressing cell susceptible to killing. Islet cells from recently diagnosed type 1 diabetes patients are known to exhibit upregulated expression of MHC class I for yet unclarified reasons. This study reports on a systematic survey of MHC class I expression patterns and potentially causal pathways in samples obtained via the network for Pancreatic Organ Donors (nPOD).

Methods: Freshly frozen pancreas samples were obtained from 39 longstanding type 1 diabetes patients, 14 non-diabetic control individuals, 5 non-diabetic, islet autoantibody positive individuals, 6 type 2 diabetes patients, 1 patient with gestational diabetes and 1 undefined case of diabetes. Sections were stained for insulin, MHC class I and CD8 by immunofluorescence. Consecutive sections from samples with pronounced MHC class I hyperexpression on islets were subjected to PCR analysis and immunofluorescence for enterovirus species and type I interferon signature genes.

Summary of Results: MHC class I hyperexpression on islets was found in four cases and was specific to type 1 diabetes. Upregulation may persist for as long as eight years after clinical onset and was observed independent of insulin sufficiency and CD8+ infiltration. Despite modulation of type I interferon signature genes, none of the samples showed evidence of chronic enteroviral infection.

Conclusions: Persistent MHC class I upregulation on pancreatic islets is a type 1 diabetes-specific phenomenon that is unlikely to be a consequence of chronic enteroviral infection.

Examination of Human Pancreatic Tissue and Isolated Pancreatic Islets from Organ Donors with Diabetes-associated Autoantibodies

Oskar Skog, Monika Hodik, and Gun Frisk
Department of Immunology, Genetics, and Pathology, Uppsala University, Uppsala, Sweden

Purpose: The notion that development of type 1 diabetes is a slow process progressing over several years is supported by the appearance of auto-antibodies against islet antigens years before clinical presentation. It is not known what triggers this humoral response, nor whether it is a cause or a consequence of the disease. In this study we had the opportunity to examine the pancreas from ten auto-antibody positive, and ten control multiorgan donors.

Methods: Insulin, immune cell marker, and enterovirus stainings were performed on formalin-fixed paraffin-embedded samples from the pancreas head. Insulin/DNA ratio, glucose-stimulated insulin release, and chemokine secretion were measured in isolated islets. PCR was used to detect enteroviral nucleic acid.

Summary of Results: Islets from pre-diabetic donors did not differ significantly from control donors in respect to immunopositivity to insulin, glucose-stimulated insulin release, or insulin/DNA ratio (3.7±2.9 in pre-diabetic and 4.9±2.0 in controls). Islet content of chemokines IL-6, IL-8, and MCP-1 was not altered in autoantibody positive donors. T cells and macrophages infiltrated the pancreatic tissue in all donors to a various extent, but no increased infiltration or accumulation in or around islets could be seen in auto-antibody positive donors compared to controls. Islets from one donor with auto-antibodies against GAD65 stained positive for enterovirus structural protein VP1 and the presence of enterovirus genome was confirmed with PCR. Inoculation of culture media from these islets on green monkey kidney cells induced cytopathic effect in these cells for up to four passages.

Conclusions: No ongoing pathogenic processes were found in pre-diabetic pancreata, but it cannot be excluded that beta-cell destruction has occurred or is ongoing in a sub-fraction of pancreatic lobules. The presence of enterovirus in pancreatic islets, at least in one of these autoantibody-positive individuals, suggests that infection can contribute to triggering the appearance of autoantibodies. This is one of very few cases in the world where the presence of enteroviral genome in islets has been proven by PCR and virus isolation.

Autoantigens, T Cells and TCRs: Human Disease-specific Therapeutic Targets

In-situ Detection of Islet Antigen-specific CD8 T Cells in Insulitic Lesions of New-onset and Long-term Type 1 Diabetes Patients

Ken T. Coppieters[1], Francesco Dotta[2], Natalie Amirian[1], Peter D. Campbell[3], Thomas W. H. Kay[3], Mark Atkinson[4], Bart O. Roep[5], and Matthias von Herrath[1]
[1]Type 1 Diabetes Center, La Jolla Institute for Allergy and Immunology, La Jolla, CA; [2]Diabetes Unit, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry, University of Siena and Fondazione Umberto Di Mario ONLUS, Siena, Italy; [3]St Vincent's Institute, Fitzroy, Victoria, Australia; [4]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL; [5]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, Netherlands

Purpose: The definition of type 1 diabetes (T1D) as an autoimmune disease has historically been inferred from the HLA associated genetic risk, islet autoantibodies and circulating beta cell-reactive T cells. However, a direct association of islet autoreactive T cells with beta-cell destruction in human pancreatic islets has never been demonstrated, while little is known about disease progression after diagnosis.

Methods: Frozen pancreas samples were obtained from eleven cadaveric T1D donors with disease durations ranging from one week to eight years. Sections were analyzed for the presence of insulin-sufficient beta cells, CD8+ insulitic lesions and HLA class I hyperexpression. Finally, consecutive sections were probed by *in situ* tetramer staining for CD8 T cell reactivity against six defined islet autoantigens associated with T1D.

Summary of Results: Pathological features such as HLA class I hyperexpression and insulitis, reported in recent-onset T1D, were found to persist in patients with longstanding disease. Insulitic lesions were present in a multifocal pattern, with varying degrees of infiltration and beta-cell loss across affected organs. Both single and multiple CD8 T cell auto-reactivities were detected within individual islets, implying that autoreactive CD8 T cells detectable in peripheral blood act locally in inflamed islets.

Conclusions: Our observations reveal a heterogeneous disease course with protracted, heterogeneous autoimmune responses in clinical T1D and provide the first direct evidence for beta cell specific CD8 T cell autoreactivity within islets. The persistence of substantial beta-cell mass and insulitis many years after clinical manifestation offers novel opportunities for therapeutic intervention, even in case of long-lasting disease.

Identification of GAD65 and Proinsulin-specific CD4+ and CD8+ T Cells in Pancreatic Lymph Nodes, Spleen, and PBMC from Subjects with T1D and Islet Autoantibody Positivity

Bilanna Hwang and Helena Reijonen
Benaroya Research Institute, Seattle, WA

Purpose: The development and progression of type 1 diabetes (T1D) is mediated by antigen-driven T cells leading to the destruction of pancreatic islet beta cells, resulting in the loss of insulin production. Antigen specific central and effector memory T cells generated in the secondary lymphoid tissues have migratory properties that allow tissue specific homing. CC chemokine receptor 7 (CCR7) and C-X-C chemokine receptor 4 (CXCR4) have been previously shown in the diabetes murine model to be important in this process. In type 1 diabetes islet autoantigen specific T cells can be identified in peripheral blood, but whether these T cells truly represent the pathogenic T cell repertoire in the pancreatic islets is still unclear. Access to pancreatic lymph nodes, spleen and PBMC from the same subjects before or around the time of onset of T1D has facilitated studies on islet specific CD4+ and CD8+ T cells that are present at the site of autoimmune inflammation. We investigated the presence of islet autoantigen specific CD4+ and CD8+ T cells in the tissue and PBMC samples from nPOD cases at different stages of disease progession: T1D (variable duration of the disease), autoantibody positive subjects (islet autoimmunity with no clinical T1D) and normal individuals (islet autoantibody negative). Cryopreserved samples from the pancreatic lymph nodes, spleen and PBMC were requested.

Methods: A comparative analysis of T cell specificities and phenotypes was performed to elucidate the differences between the T cells derived from the site of autoimmune inflammation (PLN, spleen) and periphery (PBMC) in those subjects from whom samples were available. In ex vivo tetramer assays, cells from PLN, spleen and PBMC were stained with PE labeled DR4 (DR401, 402, 404, or 405), DRB4 0101, DR301 tetramers or HLA-A2 pentamers containing GAD65, PPI and insulin peptides followed by staining with antibodies specific for exclusion and viability markers (CD14, CD19, CD56 = “dump gate”), CD3, CD4, CD8 , CD45RA/RO, CXCR4, and CCR7. Tetramer binding of the CD4+ or CD8+ T cells was analyzed by FACS-LSRII flow cytometer.

Summary of Results: GAD65 and insulin specific CD8+ and CD4+ T cells were detected in the spleen and PLN from theT1D patients and the autoantibody positive subjects and also in the PBMC from the T1D patients. Tetramer binding T cells were not detected in the tissue samples obtained from the normal individuals. All tetramer positive T cells displayed memory (CD45RO+) phenotypes, and variably expressed chemokine receptors CCR7 and CXCR4.

Conclusions: Identification of islet autoantigen specific T cells by direct tetramer staining offers an advantage in further characterizing phenotypes of T cells responding to a single, known antigen mediated by a defined MHC restriction. nPOD samples offer a unique opportunity to understand the functional and lineage specific features of migrating T cells to specific tissues in subjects at different stages of the autoimmune process. Our findings also suggest potential variability in the localization and dynamics of the islet homing T cells which remains to be further investigated.

454 Pyro-sequencing of T Cell Receptors Targeting Pancreatic Islets

Jessica Marn[1], Janet Siebert[2], Kenneth Lau[3], Hanan Ali[1], Howard Davidson[1], Peter Gottlieb[1], George S. Eisenbarth[1], Mark Atkinson[4], Joseph Larkin III[3], and Maki Nakayama[1]
[1]Barbara Davis Center, University of Colorado – Denver; [2]CytoAnalytics; [3]Department of Microbiology and Cell Science, University of Florida, Gainesville, FL; [4]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL

Purpose: The ultimate goal is to directly isolate T cell receptor (TCR) alpha and beta chain sequences from T cells infiltrating pancreatic islets of patients having type 1 diabetes (T1D) and to identify their combinations responding to islet antigens. Our first goal was to establish methodology that allows us to precisely identify "frequent" TCR sequences from limited amount of cells and tissues. Using the optimized protocol, we determined TCR sequences frequently detected in two samples from patients having type 1 diabetes.

Methods: The first RNA sample was collected from peripheral blood mononuclear cells of a T1D patient having DR4/13. We extracted RNA from the 3000 cells sorted with the gating of CD4+ and CFSE-low after a week of culture with the insulin B:9-23 peptide in the presence of IL-7 and anti-FasL Ab. The second RNA sample was obtained from the 2 x 2 inches x 20 μm of histology section generated from nPOD #6052 pancreas head. We amplified TCR alpha and beta chain DNA fragments from the RNA samples by 5'RACE PCR and ran PCR products on the 454 GSJR sequencer. To evaluate reproducibility, all the PCR products were marked by multi-identifier adaptor primers, which allow us to distinguish sequences obtained from individual PCR reactions, and were run on the 454 GSJR simultaneously. All sequences were analyzed using the IMGT-HighV-QUEST algorithm.

Summary of Results: We obtained 60,428 alpha chain and 83,224 beta chain in-framed sequences from 24 PCR reaction products generated from the 3,000 CFSE-diluted CD4 T cells. As shown in Table 1 and 2, we obtained multiple TCR sequences that are consistently frequent in multiple PCR reactions. Reproducibility among the multiple PCR reactions for both alpha and beta chains are convincing enough to identify frequent TCR chain sequences (mean R2 = 0.75 and 0.85 for alpha and beta chains respectively). Of interest, 3 out of the top 10 frequent alpha chains use the identical TRAV and TRAJ segments (TRAV14 and TRAJ5) with various junction sequences. We are currently generating Jurkat cell lines retrovirally expressing multiple combinations of these frequent alpha and beta chains to test them for response to the insulin B:9-23 peptide. Table 3 shows all the alpha chain sequences detected in 31 PCR reactions generated from the nPOD 6052 pancreatic head histology section. Although we obtained total 102,616 sequences, only 1-3 unique junction sequences were found in the individual PCR reactions. However, some of them are repeatedly detected by multiple reactions. Such sequences may frequently exist in the islets.

Conclusions: We have established the pyro-sequencing method to detect TCR sequences from the small amount of samples. Function analysis by generating T cell lines expressing frequent TCR sequences will confirm that such TCRs are truly islet-reactive and contribute to targeting pancreatic beta cells.

(Tables on next page)

Individual columns represent PCR reactions. Frequencies of TCR sequences were shown in percentage.
Dark green : >10%, Moderate green: 5-10%, Light green: 1-5%, White: not detected

Table 1: Frequent alpha chains detected in B:9-23-reactive CD4 T cells in the peripheral blood by 454 TCR sequencing with 24 PCR reactions

Vgene	Junction	Jgene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
TRAV16	CALRFGSSNTGKLIF	TRAJ37																								
TRAV8-2	CVVQRITITMTCAPSDNNNDMRF	TRAJ43																								
TRAV12-1	CVVNKGTNAGKSTF	TRAJ27																								
TRAV23/DV6	CAASGGTGGFKTIF	TRAJ9																								
TRAV38-1	CAFMSPYGGATNKLIF	TRAJ32																								
TRAV14/DV4	CAMRPMDTGRRALTF	TRAJ5																								
TRAV14/DV4	CAMRGVDTGRRALTF	TRAJ5																								
TRAV14/DV4	CAMIPMDTGRRALTF	TRAJ5																								
TRAV21	CAVKFNKFYF	TRAJ21																								
TRAV6	CALVRSTDKLIF	TRAJ34																								

Table 2: Frequent beta chains detected in B:9-23-reactive CD4 T cells in the peripheral blood by 454 TCR sequencing with 22 PCR reactions

Vgene	Junction	Jgene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
TRBV19	CASSIVGRAVDEQFF	TRBJ2-1																						
TRBV10-2	CASSERAGGSTDTQYF	TRBJ2-3																						
TRBV7-9	CASSSIWGEETQYF	TRBJ2-5																						
TRBV6-2/6-3	CASEGAGGNEQFF	TRBJ2-1																						
TRBV6-2/6-3	CASSDSSGGAGTDTQYF	TRBJ2-3																						
TRBV28	CASSGTGGGFSYTF	TRBJ1-2																						
TRBV24-1	CATSDSSGGRETQYF	TRBJ2-5																						
TRBV28	CASNSDSTGSWGQPQHF	TRBJ1-5																						
TRBV5-1	CASSETGTGKPDTQYF	TRBJ2-3																						
TRBV10-3	CAISESISPEQFF	TRBJ2-1																						
TRBV19	CASSIHGTRNTEAFF	TRBJ1-1																						
TRBV5-1	CASSPKSTSGGDNEQFF	TRBJ2-1																						

Table 3: Alpha chain sequences detected in the nPOD6052 pancreas head histology section by 454 TCR sequencing with 31 PCR reactions

Vgene	Junction	Jgene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
TRAV12-2	CAVGGAAGNKLTF	TRAJ17																															
TRAV12-2	CAVTMGSNDYKLSF	TRAJ20																															
TRAV12-3	CAMSARGGSYIPTF	TRAJ6																															
TRAV13-1	CAATPGGYNKLIF	TRAJ4																															
TRAV13-2	CAESSSYKLIF	TRAJ12																															
TRAV13-2	CAENPSGDGGYNKLIF	TRAJ4																															
TRAV13-2	CAENSGMNYGGSQGNLIF	TRAJ42																															
TRAV17	CATDASVTPLVF	TRAJ29																															
TRAV19	CALSEARSNDYKLSF	TRAJ20																															
TRAV19	CALSELSGATNKLIF	TRAJ32																															
TRAV19	CALSEAGNYGGSQGNLIF	TRAJ42																															
TRAV19	CALSEAQGGRRALTF	TRAJ5																															
TRAV21	CAVTGGNKLTF	TRAJ10																															
TRAV21	CASDSGSARQLTF	TRAJ22																															
TRAV21	CAVKPPVGGGKLIF	TRAJ23																															
TRAV21	CAVKSNSGNTPLVF	TRAJ29																															
TRAV21	CAAYSTGKLIF	TRAJ37																															
TRAV22	CAALYGNKLVF	TRAJ47																															
TRAV23/DV6	CAATPGRSGGYQKVTF	TRAJ13																															
TRAV26-1	CIVRVENQGGKLIF	TRAJ23																															
TRAV26-2	CILGENQAGTALIF	TRAJ15																															
TRAV26-2	CILNSGNTPLVF	TRAJ29																															
TRAV27	CAGKDTNAGKSTF	TRAJ27																															
TRAV38-1	CAFRVDSSYKLIF	TRAJ12																															
TRAV38-2	CAYFSGTYKYIF	TRAJ40																															
TRAV38-2	CAYRSGSNNDMRF	TRAJ43																															
TRAV4	CLVGDPSTGGAGNMLTF	TRAJ39																															
TRAV4	CLVGPLMFSGGYNKLIF	TRAJ4																															
TRAV41	CAVSFGNEKLTF	TRAJ48																															
TRAV41	CAEFYF	TRAJ49																															
TRAV6	CALDPSGGSYIPTF	TRAJ6																															
TRAV6	CAVGDPSFGNEKLTF	TRAJ48																															
TRAV8-6	CAPSPGGYNKLIF	TRAJ4																															
TRAV8-6	CAVSDMGTYKYIF	TRAJ40																															
TRAV9-2	CALSPDYKLSF	TRAJ20																															
TRAV9-2	CALSDRANAGKSTF	TRAJ27																															
TRAV9-2	CALRGSSGYELNF	TRAJ41																															

Monoclonal Expansions of TCR in a Diabetic Pancreas

Cristina Xufré, Carlos Martínez-Torró, Lorena Usero, Eduard Vico, Eloi Parladé, Eva Codina-Busqueta, Dolores Jaraquemada, Carme Roura-Mir, and Mercè Martí
Laboratory of Cellular Immunology, Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona (UAB), Spain

Purpose: T1D is an autoimmune disease characterized by the selective destruction of insulin-producing cells located within the Langerhans islets. Pathology is associated with CD4+ and CD8+ T cells infiltration around the pancreatic islets. The study of the TCR repertoire is an indirect measure of the T cell diversity that is taking part of the effector and regulatory immune response inside the islets. Several investigators have described the TRBV family repertoire of the NOD mouse intrapancreatic infiltrates from but not in humans, where the limited availability of affected-pancreatic tissue has been a handicap to performe such studies. We had the opportunity to study the TRBV repertoire of intrapancreatic T cells of a diabetic pancreas at onset (case 1) that was characterized by Somoza et al (J. Immunol 1994; 153: 1360) (Codina-Busqueta et al. J. Immunology 2011; 186: 3787).

Methods: TCR analysis was performed by multiplex RT-PCR analysis. PCR products were directly analyzed by spectratyping. Some of the products were further cloned and sequenced.

Summary of Results: Five monoclonal expansions were identified and their CDR3 sequenced: Vb1 (CASSVSTTDTQYF), Vb7 (CASSQVAGAGTGELFF), Vb11 (CASSDPGTQETQYF), Vb17 (CATSPLGMNNEQFF) and Vb22 (CASSEAQQGYSGELFF). All these clones were also expanded in the total digested tissue sample except for the Vb11 clone that was only found at low frequency, not showing a pattern of monoclonal expansion. When further analyzing the Vb11 TRBV family, using total digest, spleen and PBMCs from the same patient, we found that the distribution of Vb11 peaks did not show a normal pattern, because only a few dominant peaks with similar area were visible, corresponding to CDR3 sizes of 10, 11, 12, 14 and 16aa, respectively. Of 34 sequenced clones, only six different sequences were identified, all at a frequency range of 9-26%, without any evidence of one dominant sequence. This was not the case for any other TRBV family, where a normal distribution of CDR3 sizes was always found, with several sequences for each CDR3 size. All Vb11 peaks present in the pancreas sample but not detectable in the islets, were also detected in the spleen of the same patient, but the monoclonal 12aa CDR3 clone identified in the islets has so far not been sequenced from any of the spleen samples analyzed.
The T cells isolated from the total digest were also expanded and oligoclonal lines were generated. A large number of Vb11+ positive cell lines could be isolated, many of which shared one of the sequences found in the pancreas, but not the expanded clone from the islets. This sequence was not found in the patient's spleen.

Conclusions: These data suggest that in this particular case of T1D, the Vb11 family of TRBV can be relevant for the disease, showing an unusual pattern of CDR3 size distribution, with several dominant expansions, some of which appear to be directly involved in islet recognition. The mechanisms involved and the functional relevance of these cells are under study.

Autoreactive B Cells from Spleen and Pancreatic Draining Lymph Nodes from Type 1 Diabetes Subjects

Alexis J. Torres[1], Elizabeth M. Bradshaw[2], Adebola O. Ogunniyi[1], David A. Hafler[3], J. Christopher Love[1], and Sally C. Kent[4]

[1]Department of Chemical Engineering, The David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA; [2]Center for Neurologic Diseases, Department of Neurology, Brigham and Women's Hospital, Boston, MA; [3]Departments of Neurology and Immunology, Yale University, New Haven, CT and The Eli and Edythe L. Broad Institute, Cambridge, MA; [4]Division of Diabetes, Department of Medicine, University of Massachusetts Medical School, Worcester, MA

Purpose: The purpose of this study is to determine the frequency and function of autoreactive B cells from the spleen and pancreatic draining lymph nodes (PLN) from subjects without Type 1 diabetes (T1D), with autoantibody, but without disease and from recent and long-term T1D subjects.

Methods: The analysis of multiple secreted products from single B-cells was detected by microengraving. PLN cells or splenocytes were polyclonally stimulated with BCR cross-linking, soluble CD40L and pokeweed mitogen for 18 hours and then dispersed into the wells (100,000/slide). Glass slides are coated with antigen (rGAD65 or proinsulin) and blocked. The supernatant of the nanowells is exposed to the antigen-coated surface of the glass slide for 2 hours and antibody from the supernatant bound to the antigen is detected with anti-human Ig isotype antibodies labeled with different flurochromes and read in a microarray reader. Cells in the wells are stained with anti-CD20 flurochrome labeled antibody and imaged. Percentages of CD20+ B cells secreting autoreactive antibodies of different isotypes and subisotypes was calculated. In order to detect B-cells secreting cytokines upon polyclonal stimulation, standard intracellular cytokine staining (ICS) and co-staining with CD20+ cells was done with PLN or spleen cells. Standard quantitative PCR for cytokine message expression was done on negatively isolated CD20+ B cells.

Summary of Results: The range of autoreactive B cells was from undetectable to 0.006-0.48% of polyclonally stimulated CD20+ B cells. From 2 PLN and spleen samples from subjects without diabetes, no GAD65- or proinsulin-reactive B cells were detected. nPOD case 6044 and case 6090 are subjects who were positive for GADA in the circulation at the time of demise, but without a history of T1D. From nPOD 6044 PLN, 0.017% of CD20+ B cells secreted IgM antibody reactive with GAD65; no secreted antibody reactive with proinsulin from these two samples or secreted antibody reactive with GAD65 from case 6090 were detected. From 3 subjects with recent onset of T1D (<5 years of diagnosis), secreted antibody reactive with GAD65 (IgM, IgG1 and IgG3) ranging from 0.006 to 0.48% of CD20+ B cells was detected; secreted antibodies reactive with proinsulin were detected from the spleen and PLN from these recent T1D onset subjects. From the spleen from 4 long term (>10 yrs from diagnosis) T1D subjects, frequencies of secreted GAD reactive antibody in the range from undetectable to 0.006% to 0.1% were seen. Polyclonally stimulated CD20+ B-cells from PLN examined for cytokine expression by quantiative PCR and by ICS, a peak of expression of IL-6, IL-10, LTalpha, and TNFalpha at Day 2 post stimulation was seen.

Conclusions: Splenic and PLN CD20+ B-cell secreting antibodies reactive with GAD65 and proinsulin were detected. A trend towards an increased frequency of B cells secreting IgM, IgG1 and IgG3 antibodies reactive with GAD65 was seen from the spleen and PLN from recent onset T1D subjects as compared to the frequencies seen from these tissues from controls, long-term T1D subjects and those with serum autoantibodies, but without disease. Proinflammatory cytokine expression was detected from CD20+ PLN B cells at peak after 48 hrs of polyclonal stimulation. These studies will aid in defining the frequency/function of autoreactive B cells in human T1D and in responder and non-responder patient groups in therapies in which B cell populations are manipulated.

Enhancement of Treg-mediated Supression by NKT Cells in T1D

Lorena Usero[1], Cristina Xufré[1], Joan Verdaguer[2], Dolores Jaraquemada[1], Mercè Martí[1], and Carme Roura-Mir[1]

[1]Laboratory of Cellular Immunology, Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona (UAB), Spain; [2]Immunology Unit, Department of Experimental Medicine, Universitat de Lleida (UdL), Spain

Purpose: Type 1 Diabetes (T1D) is an organ-specific autoimmune disease characterized by the autoreactive T cell-mediated destruction of insulin-producing beta cells of the pancreas. T cells with regulatory functions, including CD4+CD25++Foxp3+ regulatory T cells (Tregs) and invariant natural killer T cells (iNKT) are important in controlling pathogenic autoreactivity. Both populations are reduced in numbers or have compromised functions in several human autoimmune diseases, including T1D. Previous data from our laboratory have described a differential distribution of iNKTs and Treg cell populations, in the pancreas of a T1D patient at disease onset. Namely, all Treg cells were found inside the islets together with a small number of NKTs, whereas the remaining NKT cells occupied the periinsular area. A very similar distribution pattern was found by immunofluorescence analysis on pancreas from diabetic NOD mice (20 weeks), that was not evident in prediabetic (9 weeks) or disease onset (14 weeks) mice, where the NKT cells were well outside the insular area. We have proposed an active cross-talk between these two types of regulatory T cells in the target organ of autoimmune disease. Our aim is to analyze their functional interaction in the pancreas of T1D patients.

Methods: To study the possible interaction between these two populations of immunoregulatory T cells, we selected iNKT and Treg cells from healthy donors PBMCs, based on the expression of CD3 and CD56 and expanded *in vitro* with CD1d+ APCs pulsed with αGalactosylCeramide (αGalCer) obtaining a population of 99,6 % of Vα24Jα18CD3 iNKTs cells. Tregs were seleted by their expression of CD4 and CD25hi and were expanded *in vitro* with anti-CD3 and anti-CD28 coated beads using the Rapid Expansion Method (REM). With this method, we obtained 99% pure CD25++CD3+CD4+Foxp3+ Tregs cells. We then analyzed the capacity of the iNKTs to modify the suppression of T effector cells (Tef) *in vitro* proliferation by Tregs, by measuring H3-thymidine incorporation after 4 days of culture, using different ratios of Treg:Teff cells. After that, we analyzed the capacity of the iNKTs to modify the suppressor effect of Treg cells by adding different numbers of iNKT cells to the same cultures.

Summary of Results: The results from NOD mice showed that both cell types are present at the diabetic pancreas but they localize inside the islets following a sequential pattern: Tregs associate preferentially to pancreatic islets even in non-diabetic pancreas while NKT cells enter the islets later in the development of the autoimmune response. iNKTs and Tregs can be expanded *in vitro* maintaining their phenotype and functionality. Treg cells with the capacity to suppress T effector cells by an average of 36% increased the effect to a 60% after the addition of NKT cells to the assay. We are investigating the mechanism by which NKT cells enhance the suppressor effect by addressing the requirement of cellular contact and the production of inhibitory soluble factors.

Conclusions: NKT cells have an adjuvant effect on Tregs by increasing their capacity of inhibiting Tef proliferation *in vitro*. The co-localization of these two cell populations in the diabetic pancreas suggest that this effect could be taking place *in vivo*. If confirmed in human, this adjuvant effect could represent a tool to overcome the proposed resistance of diabetic Tef cells to supression by Tregs.

Heterogeneity of Pancreas Pathology and Disease Mechanisms

Expression and Regulation of Chemokines in Human Islets and Type I Diabetes

Suparna Sarkar[1], Francisco Victorino[2], Tom T. Nguyen[3], Catherine E. Lee[4], Jay A. Walters[1], Adam Burrack[2], Jens Eberlein[3], and Dirk Homann[3]
[1]Barbara Davis Center for Childhood Diabetes, University of Colorado – Denver, Aurora, CO; [2]Department of Immunology, University of Colorado – Denver, Aurora, CO; [3]Department of Anesthesiology, Barbara Davis Center for Childhood Diabetes, University of Colorado – Denver, Aurora, CO; [4]University of Colorado – Denver, Aurora, CO

Purpose: More than half of the 46 known human chemokines have been associated with and/or implicated in the pathogenesis of type 1 diabetes (T1D), yet their actual expression patterns in the islet environment of T1D patients remain at present poorly defined. This study was designed to identify relevant chemokine proteins expressed *in situ* in the pancreata of individuals with T1D to provide a foundation for the informed selection of potentially suitable targets within the chemokine/receptor family.

Methods: Here, we have employed an integrated and unbiased approach using a human islet culture system, murine models of virus-induced and spontaneous T1D, and the histopathological examination of pancreata from healthy and diabetic organ donors. Principal experimental methods included microarray analyses, qRT-PCR and immunohistochemical analyses using a set of in part newly validated reagents.

Summary of Results: CCL5, CCL8, CCL22, CXCL9, CXCL10 and CX3CL1 were the major chemokines transcribed (in an iNOS- but not NF-κB-dependent fashion) and translated in response to inflammatory stimuli. CXCL10 was identified as a dominant chemokine expressed at the protein level in the islet environment of both experimental animals and T1D patients, while CCL5, CCL8, CXCL9 and CX3CL1 were expressed at lower levels in murine and human T1D. Unexpectedly, these chemokines, and in particular CXCL10, were also expressed in the acinar tissue of the exocrine pancreas.

Conclusions: The utility of our integrated screening approach was validated by identification of CXCL10 as a particularly prominent chemokine expressed in the pancreata of T1D patients, and further provides evidence for the potential importance of CCL5, CCL8, CXCL9 and CX3CL1 in the pathogenesis of human T1D. Importantly, expression of these chemokines in both islets and acinar tissues emphasizes an underappreciated involvement of the exocrine pancreas in the natural course of T1D that will require consideration for further T1D pathogenesis and immune intervention studies.

Loss of Beta Cell Heparan Sulfate and Expression of Heparanase by Insulitis Mononuclear Cells Correlates with Type 1 Diabetes in Human Pancreas Specimens

Lora Jensen[1], Andrew Ziolkowski[1], Sarah Popp[1], J. Dennis Wilson[2], Christopher Parish[1], and Charmaine Simeonovic[1]

[1]Department of Immunology, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia; [2]Department of Endocrinology, The Canberra Hospital, Woden, ACT, Australia

Purpose: Our studies have shown that heparan sulfate (HS), a glycosaminoglycan or complex sugar, is expressed strongly by mouse islets *in situ* and plays a critical role in maintaining beta cell survival. In NOD/Lt mice, T1D development correlates with progressive loss of HS in beta cells and expression of heparanase (Hpse; an endoglycosidase that degrades HS) by insulitis mononuclear cells (MNCs). Treatment of NOD/Lt mice with a Hpse inhibitor (PI-88) can prevent T1D, protect islets from destructive autoimmunity and preserve beta cell HS *in situ*. We therefore propose that beta cell HS and Hpse play critical roles in beta cell heath and T1D disease, respectively. This study investigated whether like mouse islets, normal human islets *in situ* characteristically show intense localisation of HS in beta cells. In addition, we examined whether the development of T1D in humans is associated with (i) loss of islet-associated HS, and (ii) expression of Hpse by insulitis MNCs.

Methods: HS immunohistochemistry was performed on normal (#6075, #6134, # 6096, #6094), T1D (#6052, #6062, #6069, #6045, #6145), autoantibody+ve T1D-ve (#6080, #6027, #6044, #6090) and T2D (#6108, #6109, #6059, #6028) pancreas samples. Following antigen retrieval using 0.05% pronase, sections were stained using mouse 10E4 mAb (Seikagaku) that recognises highly sulfated HS, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig and 0.04% 3-amino-9-ethylcarbazole (AEC)/0.015% hydrogen peroxide. Mouse IgM was used as the isotype control. Quantitative analyses of the intra-islet area of 10E4+ve staining was done using Image J software with color deconvolution plug-in (n=4/group for normal, T1D or T2D pancreas specimens, 10 islets examined/specimen). For antigen retrieval and Hpse immunohistochemistry, sections were initially heated in citrate buffer (pH 6.0) and then stained using HP130 mouse anti-Hpse mAb (Insight), the PK-2200 M.O.M. Immunodetection kit (Vector Labs) and AEC/0.15% hydrogen peroxide. Mouse IgG1 was used as the isotype control. For insulin and glucagon immunostaining, mouse anti-insulin and mouse anti-glucagon mAbs (Sigma) were used with the M.O.M. kit.

Summary of Results: HS was strongly expressed in normal human islets *in situ* and correlated with the immunolocalisation of insulin+ve beta cells and not glucagon+ve alpha cells, suggesting that the HS was localised in islet beta cells. Intense HS staining was similarly found in islets of autoantibody+ve T1D-ve pancreas samples. In contrast, islets from T1D specimens, showed low or no intra-islet staining for HS even in the presence of strong insulin staining (#6052 and #6069). Quantitative analyses revealed a 10-fold reduction in the HS content of T1D islets compared to normal islets ($P<0.0001$) and a 3-fold reduction in T2D islets ($P<0.0001$). Staining for Hpse was found to be intense on insulitis MNCs and prominent in some islets in T1D specimen #6052 (at 1 year post-onset). In contrast, Hpse staining was variable in normal human islets with weak intracellular or cell surface localisation.

Conclusions: These findings indicate that progression of T1D disease in humans correlates with loss of beta cell HS, probably due to the production of Hpse by insulitis MNCs and possibly by the beta cells. Loss of HS in islet beta cells may represent a common pathway leading to the decline in beta cell viability for both T1D and T2D.

Specific Extracellular Matrix Components Accumulate in Islets and Lymphoid Tissues of Type 1 Diabetic Patients

Marika Bogdani[1], Susan Potter-Perigo[2], Paul L. Bollyky[3], Pamela Y. Johnson[2], Gerald T. Nepom[3], and Thomas N. Wight[2]

[1]Pacific Northwest Diabetes Research Institute, Seattle, WA; [2]Hope Heart Matrix Biology Program, Benaroya Research Institute at Virginia Mason, Seattle, WA; [3]Diabetes Research Program, Benaroya Research Institute at Virginia Mason, Seattle, WA

Purpose: The process of β cell destruction in T1D relies on migration of inflammatory cells from the blood stream into pancreatic islets via interaction with the extracellular matrix (ECM). We examined the role of ECM molecules in creating a permissive environment for autoimmune attack, focusing on hyaluronan (HA), an ECM glycosaminoglycan. Our study aims to 1) determine the changes that occur in the amount and distribution of HA in the ECM of T1D pancreatic islets and lymphoid tissues, and 2) examine the HA-dependent binding capacity of T cells to the ECM and whether it is altered in T1D.

Methods: Pancreas and lymphoid tissue sections from T1D patients and age-matched controls were provided by nPOD. HA presence and localization were examined by affinity histochemistry and HA accumulation determined semi-quantitatively. T cells, freshly isolated from T1D subjects and controls, were cultured on ECM enriched in HA to determine their HA-binding capacity and examined for the expression of the HA receptor CD44v6.

Summary of Results: In both T1D and control pancreatic tissues, HA was present in islets, associated with the islet capillaries. The relative and total intra-islet HA positive area varied among samples. Two of 12 T1D tissues contained islets with insulitis. Intra-islet HA staining was markedly elevated in these tissues which contained inflammatory cells that were embedded in this HA-enriched ECM. Evaluation of HA involvement in the islets of two different animal models of T1D, the NOD mouse and BB rat, revealed a similar distribution of HA associated with inflammatory cell infiltrates in the islets. To examine whether similar ECM changes occurred in T and B cell germinal centers (GC), HA staining was performed in pancreatic lymph nodes and spleens from T1D and control donors. Results indicated that HA was present in the T cell areas of lymph nodes and spleens in the controls. However, this staining was markedly intensified in the T cell compartments and in the enlarged GC of B cell follicles in the T1D tissues. 58% of follicles in the T1D spleen accumulated HA, occuping more than 50% of the GC area, while only 14% of follicles in the non-diabetic spleen were HA positive. Similarly, in contrast to control lymph nodes in which only 16% of follicles showed weak HA staining, 96% of T1D follicles presented moderate to strong HA staining covering more than 80% of their area. These results indicate that major ECM changes also occur in tissues other than islets in T1D. In an *in vitro* assay, CD4 T cells adhered to an HA-enriched ECM in a hyaluronidase-sensitive manner. HA binding and clearance are mediated by CD44, particularly, the CD44v6 isoform. Upon activation, CD4 T cells isolated from T1D subjects expressed less CD44v6 relative to healthy controls, suggesting that HA may accumulate in follicles of T1D patients due to impaired CD44v6-mediated HA turnover and these differences may influence the interaction of T cells with HA-enriched matrices.

Conclusions: Our preliminary data indicate that HA accumulates in islets and lymphoid tissues of T1D patients. HA occurs in close physical association with infiltrating leukocytes in insulitis and amasses in T cell areas and enlarged GC in lymphoid follicles, both in human and rodent T1D tissues, indicating the participation of HA in the inflammatory processes in T1D. Work is in progress to further clarify the role HA may play in these processes.

Down Regulation of Glucokinase and Mitochondrial ATP Synthase in Islets from Type 1 Organ Donors

Yaima L. Lightfoot[1], Scott Grieshaber[2], Jason Wilhem[1], Li Zhang[1], Martha Campbell-Thompson[1], Desmond Schatz[3], Mark Atkinson[1], and Clayton E. Mathews[1]
[1] Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL; [2]Department of Oral Biology, University of Florida, Gainesville, FL; [3]Department of Pediatrics, University of Florida, Gainesville, FL

Purpose: Glucose metabolism and the resulting ATP production are essential for stimulus coupled insulin secretion and maintenance of normal blood glucose levels. Using human pancreatic organ samples obtained from the Network for Pancreatic Organ donors with Diabetes (nPOD) initiative, we sought to identify differences in essential components of glucose stimulated insulin secretion (GSIS) in individuals with Type 1 diabetes (T1D) and individuals at risk for T1D, as determined by circulating autoantibody positivity (AAb+/non-T1D), compared to T1D free controls.

Methods: Slides with tissues from the following groups were requested from nPOD: T1D free controls (n=4), AAb+/non-T1D individuals (n=6), and T1D subjects with insulin positive (INS+) beta cells remaining within the islets (n=4). Interestingly, approximately one-third of the organs from patients with long-standing T1D in the nPOD collection have a significant number of INS+ islets. Samples were age and sex-matched. Slides were stained for insulin (INS), as well as for glucokinase (GCK) and the β subunit of the F1Fo ATP Synthase (ATPase), two essential proteins for GSIS by β cells. A Zeiss Axioskop Microscope was used to capture images that were subsequently analyzed using ImageJ/Fiji (NIH). Microscope and camera settings were identical for each islet on every slide. Normalizing protein expression levels by exocrine staining minimized the contributions of potential differences in staining from sample to sample due to their specific collection and preparation. We compared the mean pixel intensity of GCK and ATPase in INS+ endocrine cells to their expression in the exocrine tissue, and determining differences, if any, between the groups tested.

Summary of Results: The expression of ATPase was decreased in the INS+ cells of T1D patients, compared to those in AAb+/non-T1D or T1D free samples. GCK was significantly reduced in the INS+ cells of both T1D+ and AAb+/non-T1D samples compared toT1D free sections. GCK levels were identical when comparing INS+ cells from T1D+ samples to AAb+/non-T1D samples. Further, the staining intensities of both GCK and ATPase in INS+ cells correlated to c-peptide levels in the circulation of the organ donors [GCK: $R^2=0.7$ & $p=0.02$, and ATPase: $R^2=0.58$ & $p=0.048$]. The staining intensity of insulin within the beta cells did not differ between the three groups and also did not correlate with circulating C-peptide in the organ donors.

Conclusions: Insulin positive beta cells are present in a number of the T1D cases in the nPOD collection despite reduced beta cell mass. In addition, deficiencies in GCK and the mitochondrial ATPase in T1D+ subjects may be responsible for decreased beta cell function. This suggests that in patients with long-standing diabetes, beta cells are present but function is impaired. Understanding this process could have a major impact on how T1D is treated. The reduction in GCK in the AAb+/non-T1D cases, may demonstrate the presence of the ongoing T1D processes at the beta cell level.

Complement Activation in Type 1 Diabetes: Analysis of Pancreatic Tissue from nPOD Cases

Patrick Rowe[1], Clive Wasserfall[1], Byron Croker[1], Martha Campbell-Thompson[1], Desmond Schatz[2], and Mark Atkinson[1]
[1]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL; [2]Department of Pediatrics, University of Florida, Gainesville, FL

Purpose: Decades ago, antibody-mediated complement activation was considered to have a pathogenic role in type 1 diabetes (T1D). Despite this, interest in the potential pathogenic role for autoantibodies and B lymphocytes in T1D declined. However, recent studies have brought the focus back to a role for humoral immune mechanisms (eg, gene associations with several complement proteins, therapeutic interventions with B lymphocyte-directed agents, etc). Therefore, we investigated whether evidence of complement activation could be found in pancreatic tissue from organ donors with established T1D and/or donors without diabetes positive for one or more islet autoantibodies (mIAA, IA-2, GAD65, ZnT8), thought to represent the early stage of the disease.

Methods: Immunohistochemical (IHC) techniques were used to measure density of the complement activation product C4d (mouse α-human C4d IgG1, clone 10-11) in pancreata from the different donor groups [no diabetes (n=11), T1D (n=11), no diabetes autoantibody-positive (n=5)]. IHC staining was ranked by a pathologist blinded to the donor groups and C4d density was quantified by analyzing whole-section images from digitally scanned slides using ImageScope software.

Summary of Results: Regardless of donor group or staining density, C4d immunoreactivity was primarily restricted to blood vessels. C4d density was significantly higher, as assessed both by pathologist ranking (T1D: 21.3±1.4, no diabetes: 8.5±1.2; mean±SE, $p<0.001$) and computer-aided image analysis (T1D: 25.2±4.1%, no diabetes: 2.1±0.5%; mean±SE, $p<0.001$), on pancreatic sections from donors with T1D compared to donors without diabetes. No significant differences were found between autoantibody-positive (rank: 10.2±3.7, density: 2.7±1.3%) and autoantibody-negative nondiabetic donors.

Conclusions: Our results suggest that complement activation is occurring via the classical (antibody-mediated) pathway within pancreatic tissue from long-standing T1D donors, a finding that may be related to the persisting pro-inflammatory environment in T1D pancreata, vascular effects of long-term hyperglycemia, or donor group differences in acute responses to stress-hyperglycemia just prior to death. Future analyses will focus on colocalizing approaches to determine whether C4d-positive blood vessels are associated with islets in specific donors and/or donor groups, C4d immunostaining on additional nPOD cases with type 2 diabetes to serve as hyperglycemia controls, and measures of average blood glucose (fructosamine).

Adhesion Molecules on High Endothelial Venules of Pancreatic Lymph Nodes from Humans with Type 1 Diabetes

Elle Glueckert[1], Baohui Xu[2], and Sara Michie[3]
[1]Michigan State University, College of Medicine, East Lansing, MI; [2]Department of Vascular and Endovascular Surgery, Stanford University School of Medicine, Stanford, CA; [3]Department of Pathology, Stanford University School of Medicine, Stanford, CA

Purpose: In the nonobese diabetic (NOD) mouse model of type 1 diabetes, naive autoreactive T cells migrate through high endothelial venules (HEVs) into pancreatic lymph nodes (PanLNs), where the T cells are primed by β cell antigens. Progeny of the primed T cells migrate from blood vessels into pancreas, leading to development of islet inflammation, β cell destruction and overt diabetes. We have shown that the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is strongly expressed on HEVs of PanLNs from young NOD mice and plays a major role in migration of naive autoreactive T cells into PanLNs. In contrast, the peripheral node addressin (PNAd) shows various levels of expression on HEVs of PanLNs from young NOD mice and plays a minor role in migration of naive autoreactive T cells into PanLNs. The HEV adhesion molecules that mediate the migration of T cells from the bloodstream into PanLNs of humans are not known.

Methods: We used immunohistology staining to determine which adhesion molecules are expressed on HEVs of PanLNs from humans with type 1 diabetes.

Summary of Results: We found that most PanLN HEVs in humans, as in NOD mice, had:
1) strong expression of MAdCAM-1; and
2) variable expression of PNAd, with some HEVs showing strong diffuse staining and others showing weak and/or focal staining.

Conclusions: These results suggest that the HEV adhesion molecules that mediate T cell migration into PanLNs in the initiation stage of the autoimmune response in NOD mice might also mediate T cell migration into human PanLNs. This supports the use of NOD mice in translational research on the lymphocyte migration pathways that are involved in the development of human type 1 diabetes.

Revelation of Altered Expression of B7-H4: Decrease in Type 1 Diabetes and Increase in Insulinoma

Susan S. C. Cheung[1], Dawei Ou[1], Daniel L. Metzger[2], Ziliang Ao[1], David Owen[3], and Garth L. Warnock[1]
[1]Department of Surgery, University of British Columbia, Vancouver, British Columbia, Canada; [2]Department of Pediatrics, University of British Columbia, B.C.'s Children's Hospital, Vancouver, British Columbia, Canada; [3]Department of Pathology and Lab Medicine, University of British Columbia, Vancouver General Hospital, Vancouver, British Columbia, Canada

Purpose: B7-H4 is a novel negative T-cell co-signaling molecule. Our study results indicate that B7-H4 shows weaker expression levels in the pancreatic islet β cells from both human autoantibody-positive cadaver donors (IAPD) and type 1 diabetes (T1D) patients, compared with those from normal controls. Moreover, our research shows that B7-H4 is co-expressed with insulin in the islet β cells, and that its expression is concomitantly reduced with insulin in the pancreas of T1D patients. In contrast, increased B7-H4 protein expression has recently been found in a variety of human cancer cells, including lung, ovarian, breast, pancreas, brain, stomach, uterine, and kidney. We study here the expression of B7-H4 and insulin in the 'normal'-appearing islets and β cell adenomas of pancreatic samples from patients with insulinoma in comparison with those of islets from patients with T1D and from normal controls.

Methods: Ten archival insulinoma pathology samples were studied by bright-field immunohistochemistry (IHC) for the B7 family of molecules (B7-H1, -H2, -H3, -H4) as well as for insulin. Ten samples each from normal and T1D pancreas sections were included for comparison. Multi-fluorescence IHC was used to study marker expression level [mean fluorescence intensity (MFI) of positive cells and co-localization of markers (expressed as Pearson's correlation coefficient r)]. B7-H4 mRNA transcripts and protein were determined by qRT-PCR and Western blot assay, respectively.

Summary of Results: All insulinoma samples show moderate B7-H4 and strong insulin expression by bright-field IHC in the β cells from both the islets and adenomas. Co-expression of B7-H4 and insulin in the islet and adenoma β cells is reduced, respectively, at 0.66 ± 0.03 (N=19, $p<0.0001$) and 0.51 ± 0.03 (N=19, $p<0.0001$), compared to the normal control islets at 0.83 ± 0.01 (N=78), and T1D islets at 0.45 ± 0.03 (N=43, $p<0.0001$). Levels of B7-H4 and insulin expression are higher in islet and adenoma β cells than those of normal controls: B7-H4 cellular MFI in the insulinoma islet and adenoma β cells are, respectively, 55.77 ± 2.64 (N=19, $p<0.0001$) and 53.02 ± 3.83 (N=19, $p<0.0001$), compared to that of normal controls at 30.77 ± 1.11 (N=78); while insulin MFI in the insulinoma islet and adenoma β cells are, respectively, 88.35 ± 4.16 (N=19, $p<0.0001$) and 82.8 ± 3.88 (N=19, $p<0.0001$), compared to that of normal controls at 55.74±0.99 (N=78).

Conclusions: This study has shown that β-cell B7-H4 expression is deceased in T1D and increased in insulinoma patients. This suggests that B7-H4 may be involved in the pathogenesis and development of these diseases, possibly through breakage of immune tolerance to β cells in T1D or—in contrast—through facilitating malignant cell evasion from immunosurveillance in insulinoma.

From Genetics to Gene Expression in nPOD Tissues

Disease-dependent and Tissue-specific Changes in Gene Expression in Type 1 Diabetes (T1D)

Chan C. Whiting, Jill Schartner, and C. Garrison Fathman
Division of Rheumatology and Immunology, Department of Medicine, Stanford University School of Medicine, Stanford, CA

Purpose: Use microarray global gene expression profiling to compare mRNA expression in different tissues (pancreatic lymph node, spleen) from T1D patients and normal subjects to identify genes whose expression is disease-dependent and tissue-specific.

Methods: To identify differentially expressed genes in T1D verses healthy controls, we performed cDNA microarray analysis on pancreatic lymph node (pLN) and splenic tissues from 10 T1D patients and 10 healthy controls. These tissues were snap frozen in RNAlater and total RNA was extracted using standard Trizol methods. All frozen tissue samples were kindly provided by nPOD. Gene expression was analyzed using an Agilent 41K Whole Human Genome (60-mer) Oligo Microarry Kit. Statistical and pathway analyses of differentially modulated genes in T1D were performed using Genespring and Ingenuity Pathway Analysis (IPA) software tools, respectively.

Summary of Results: Large-scale gene expression analysis of T1D patients' tissues compared to healthy controls revealed disease-dependent and tissue-specific modulation of genes. Despite the heterogeneity in global gene expression in individual samples, disease-dependent 1532 entities with over annotated 1000 genes were specifically modulated by at least 1.5X in pLN of T1D patients while only 459 entities or slightly over 300 genes were changed in the spleen. These disease-dependent modulated genes were also tissue-specific since there is little overlap between genes differentially expressed in the pLN and spleen. Most differentially expressed genes in the spleen are non-immune specific while those in the pLN are enriched for a network of immune- and inflammation-related genes that clearly distinguish T1D from healthy individuals. These data question the notion raised by SNP and GWAS studies proposing that specific fixed mutations in disease-relevant genes are a major cause of T1D. Rather than global genetic defects in all tissues and cells as SNPs and GWAS mutations would propose, our data support the concept that disease-specific changes in gene expression occur in specific tissues such as the pLN. In particular, the genes expressed differentially in the pLN are involved in regulatory pathways including MHC molecules, NFkB, TCR, apoptosis, PI3K and Stat proteins whose expression is altered in T1D compared to controls. Remarkably, a significant number of these genes are seen in in cytotoxic T cell mediated apoptosis, antigen presentation, OX40 signaling and cell cycle regulation.

Conclusions: Global gene expression analysis of T1D patients versus healthy controls revealed disease-dependent and tissue-specific gene signatures. Key changes in the expression of genes relevant to immune and inflammation-specific pathways were seen in the pLN. Overall, tissue specific gene expression in T1D was primarily associated with cell cycle, immune regulation, transcriptional regulation and signal transduction. These novel gene signatures and their role in immune regulation may provide insights into the pathogenesis of T1D and possibly provide targets for immunotherapy.

Identification of Extra-thymic Aire-expressing Cells (eTACs) in Human Tissue and Comparison between Type-1 Diabetic and Non-diabetic Controls

Eileen J. McMahon[1], Todd C. Metzger[2], and Mark S. Anderson[2]
[1]Westmont College, Santa Barbara, CA; [2]Diabetes Center, University of California – San Francisco

Purpose: Autoimmune regulator (Aire) was identified in 1997 as the gene mutated in autoimmune polyendocrine syndrome type I (APS-1), a disease in which patients develop a wide range of autoimmune diseases including type I diabetes (T1D). Aire is known to be highly expressed in medullary thymic epithelial cells where it upregulates tissue-specific antigens (TSAs) for negative selection of T cells. Recently, using transgenic reporter mice, our lab has characterized a rare population of cell in secondary lymphoid organs that also expresses Aire. Furthermore, these eTACs (extra-thymic Aire-expressing cells) could promote peripheral tolerance to prevent autoimmune diabetes in an antigen-specific manner. The goal of this study was to identify and characterize eTACs in human lymph node and spleen and to compare eTACs in T1D samples and healthy controls.

Methods: Immuno-fluorescent staining with anti-Aire antibody was used to identify eTACs in human pancreatic and non-pancreatic LN (PLN and nPLN, respectively). Sections were also co-stained for MHC class II and CD11c, a prototypical dendritic cell marker. RNA was isolated from frozen whole PLNs and from sorted populations from fresh spleen. Quantitative PCR was used to measure relative Aire expression and to identify potentially Aire-regulated TSAs.

Summary of Results: Aire+ cells were readily identified by immuno-staining in both PN and nPLN sections. Like in mouse, human eTACs were relatively rare but localized outside the B-cell follicles and stained with the hallmark “nuclear speckling” pattern. Furthermore, the eTACs were ubiquitously positive for MHC class II but lacked high expression of CD11c, calling into question their identification as a known resident DC population. Quantitative PCR confirmed the expression of Aire in whole PLN and in sorted CD45+, MHC class II+ populations from fresh human spleen. No difference was found in the relative expression of Aire between T1D, no diabetes, and pre-T1D PLN specimens. However, the expression of ladinin-1, a putative Aire-regulated TSA in mouse eTACS, was positively correlated with Aire expression, suggesting that it might be an Aire-regulated TSA in humans as well.

Conclusions: Overall, our data confirm that Aire is indeed expressed outside the thymus in humans and that human eTACs appear analogous to the more fully characterized murine eTACs. Their potential for promoting peripheral tolerance in a therapeutic setting remains an exciting possibility.

ImmunoChip Data on nPOD Samples

Suna Onengut-Gumuscu, Wei-Min Chen, Patrick Concannon, and Stephen Rich
Center for Public Health Genomics, University of Virginia, Charlottesville, VA

Purpose: Genome Wide Association Studies (GWAS) have contributed substantially to identification of disease susceptibility loci. A recent meta-analysis of Type 1 Diabetes (T1D) identified more than 40 T1D risk loci. Despite this advance in knowledge, the identified loci contain a median of 4 genes with a range of 0 to 27. One of the tasks ahead is to fine-map each locus to better define the likely causative SNP(s) and genes. The ImmunoChip consortium was established to design a cost effective genotyping array to fine map well established GWAS reported risk loci in immunologically related human diseases including type 1 diabetes, ankylosing spondylitis, Crohn's disease, celiac disease, IgA deficiency, multiple sclerosis, primary biliary cirrhosis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, autoimmune thyroid disease and ulcerative colitis.

Methods: A total of 186 distinct loci were targeted in the genotyping array, with all loci having at least one GWAS reported index SNP that achieved genome wide significance criteria of $P<5x10\text{-}8$ in autoimmune related diseases. SNPs included in the ImmunoChip panel were chosen from the 1000Genomes Project pilot CEU population variants, investigator contributed variants identified through disease specific resequencing projects and additional “wild card” variants. A total of 196,524 SNPs passed Illumina design metrics and were included on the ImmunoChip. On average, 643 SNPs were included in each non-HLA T1D risk locus and over 6,000 SNPs saturated the HLA region.

Summary of Results: While 69% of SNPs genotyped were common (MAF>0.05), 31% of the SNPs were infrequent (14.7% 0.05>MAF>0.01 and 16.3% of the SNPs were rare (MAF<0.01)). We have genotyped 6,741 cases and 6,622 controls along with 2,835 affected sib pair (ASP) families and 494 T1D trio families (total of 12,983 samples) previously recruited by the Type 1 Diabetes Genetics consortium (T1DGC). We have also genotyped 149 nPOD samples using the ImmunoChip arrays in order to provide a unique pathological insight for any defined risk variants identified from the T1D ImmunoChip fine mapping efforts.

Conclusions: The nPOD samples will provide a unique resource to facilitate genotype-phenotype-tissue studies to help improve our understanding of disease development.

Characterizing Gene Expression in nPOD Donor Islets

Nataliya Lenchik and Ivan C. Gerling
Department of Medicine, University of Tennessee Health Science Center, Memphis, TN

Purpose: The purpose of our study is to use laser-capture microscopy to collect islets from auto-antibody positive and control (ab negative) nPOD donors, and obtain a comprehensive islet mRNA expression profile. Comparing the two groups will allow us to gain insights into affected pathways and molecular abnormalities in islets from people in prediabetic and early diabetic stages.

Methods: We prepare slides to be RNAse free and send them to the nPOD laboratory where pancreatic tissue cryo-sections are cut onto the slides, then shipped back to us on dry ice. Slides are fixed, dried, and then laser-capture microscopy is conducted to capture 60-80 islets from each sample. RNA is immediately extracted from the tissue and stored a -80C. The quality and integrity of total RNA is tested using an Agilent Bioanalyzer 2100 system and quantified by Nano-Drop 2000. Only samples with intact ribosomal RNA peaks (18s, 28s), 260:280 absorbance ratios of 1.8-2.1, and RNA Integrity Numbers (RIN) of above 5.0 are processed further. The RNA is amplified using kits that are appropriate for the downstream transcriptome or RT-PCR experiments. Both Affymetrix expression arrays and RNA sequencing technologies are employed to obtain global gene expression signatures from autoantibody positive and negative tissue donors.

Summary of Results: We have developed methods and procedures that allows us to obtain high quality RNA from some islet donors (RIN numbers of 5.6-7.3, n=4). However from other donors the RNA was of much less quality (RIN numbers of 2.2-4.5, n=8).

Conclusions: We have demonstrated the feasibility of obtaining comprehensive gene expression signatures from islets from approximately 1/3 of the nPOD donor pancreata using laser-capture. Gene expression studies in most nPOD donor islets may require use of methods that are less sensitive to the quality of the RNA.

Peripheral Blood Monocyte Gene Expression Profile and Pancreatic Monocyte Infiltration in Patients with Type 1 Diabetes

Xiaoyu An[1], Katharine Irvine[1], Andrew Cotterill[2], Mark Harris[2], and Ranjeny Thomas[1]
[1]University of Queensland Diamantina Institute (UQDI), Princess Alexandra Hospital, Brisbane, Australia;
[2]Department of Pediatric Endocrinology, Mater Children's Hospital

Purpose: Heterogeneity in type 1 diabetes progression is poorly understood, and there is an urgent need for biomarkers stratifying disease control and outcome. We and others have observed abnormalities in peripheral blood (PB) monocytes and monocyte-derived dendritic cell (DC) activation in patients with type 1 diabetes. The aim of our nPOD investigation is to characterise pancreatic leukocytes, in particular monocytes and DC, in healthy and diabetic subjects.

Methods: We profiled PB monocyte gene expression in 6 healthy subjects and 16 children with type 1 diabetes diagnosed ~3 months previously, and analyzed clinical features from diagnosis to 1 year. Monocytes, macrophages, and subset-specific markers were stained in pancreas from 2 patients with no history of diabetes, 2 with a history of islet autoantibodies (AB+), 2 with a history of type 2, and 3 with a history of type 1 diabetes, using the nPOD tissue database.

Summary of Results: PB monocyte expression profiles clustered into two distinct subgroups, representing mild and severe deviation from healthy controls, along the same continuum. Patients with strongly divergent monocyte gene expression had significantly higher insulin dose-adjusted HbA1c levels during the first year, compared to patients with mild deviation. The PB diabetes-associated expression signature identified multiple perturbations in pathways controlling cellular metabolism and survival, including endoplasmic reticulum (ER) and oxidative stress (e.g. induction of HIF1A, DDIT3, DDIT4 and GRP78) and reduction in the CD16+ monocyte subset marker CX3CR1. In the pancreas of patients with type 1 diabetes, CD14+ monocytes were observed around the endothelium of blood vessels, closely associated with HLA-DR+ cells, CD123+ plasmacytoid DC, and varying numbers of CD3+ T cells. CD14+ monocytes and CX3CR1+, CD16+ or CX3CR1+CD16+ cells were observed frequently, and were much more numerous than CD68+ macrophages. Expression of CD16 and CX3CR1 varied between donors with T1D.

Conclusions: A PB monocyte gene expression signature correlates with glycaemic control in the first year after T1D diagnosis. These findings implicate monocyte phenotype as a candidate biomarker for disease progression after onset, and systemic stresses as contributors to innate immune function in type 1 diabetes. CX3CR1+ cells may be recruited to the pancreas in patients with T1D. Ongoing studies will correlate the level of pancreatic recruitment with PB monocyte phenotype, and markers of ER and oxidative stress in pancreatic islets of individuals with and without diabetes.

Whole Genome Scale DNA Methylation Differences in Type 1 Diabetes Disease-related Cells

Huriya Beyan[1], V. Rakyan[1], D.J. Pennington[1], J. Peng[1], T. Down[2], and Richard David Leslie[1]
[1]Blizard Institute, London, UK; [2]Gurdon Institute, Cambridge, UK

Purpose: Type 1 diabetes (T1D) is due to the interaction of genetic and non-genetic factors. The latter may influence the former through epigenetic changes including DNA methylation. Thus we have initiated 1) a discovery programme to identify DNA methylation variable positions (MVPs) in disease-relevant tissues; 2) a development programme to define MVPs in disease relevant tissues when DNA amount is limited; 3) a development programme to collect thymus and obtain DNA/RNA from it.

Methods: In an initial study we generated genome-wide DNA methylation profiles (EWAS) using Illumina 27K arrays of purified CD14+ monocytes (an immune effector cell type relevant to T1D pathogenesis) from 15 T1DM-discordant MZ twin pairs, as well as antibody positive children pre- and post-diabetes onset and antibody positive children who are now unlikely to develop T1DM plus control subjects.
In the development programme we tested three different approaches for DNA methylomics, each offering different advantages: Illumina 450K arrays, and two high-throughput sequencing (HTS)-based methods: Methylated DNA Immunoprecipitation combined with HTS (mini-MeDIP-seq); and HTS of randomly sheared, bisulfite-treated gDNA (BS-seq).
We have established a protocol for thymocyte and stromal cell isolation and good quality DNA and RNA from these paired samples from the same individual. In addition, fresh thymic tissue was mounted in preservative blocks and frozen for later use in microscopy studies and for nPOD collection.

Summary of Results: We identified 132 different CpG sites at which the direction of the intra-MZ pair DNA methylation difference significantly correlated with the diabetic state i.e. T1D-associated methylation variable positions (T1D-MVPs). We confirmed these T1D-MVPs display statistically significant disease-associated DNA methylation variation in an independent set T1D-discordant MZ pairs ($P = 0.035$). Then, to establish the temporal origins of the T1D-MVPs, we generated two further genome-wide datasets and found that, when compared with controls, T1D-MVPs are enriched in singletons both before ($P = 0.001$) and at ($P = 0.015$) disease diagnosis, and also in singletons positive for diabetes-associated autoantibodies but disease-free even after 12 years follow-up ($P = 0.0023$). The genes affected encompass: immune response pathways, notably HLA class II gene, HLA-DQB1, RFXAP, an HLA class II regulating element, NFKB1A, an important regulator of apoptosis, TNF, a key inflammatory cytokine, and GAD2 which encodes GAD65, a major T1D autoantigen. We developed a method to identify DNA methylation in small tisssue samples and found that mini-Medip-Seq gave comparable results to the other methods and only required 200 ng DNA. To date we have 10 paired samples of thymus thymocytes and stromal cells.

Conclusion: These results suggest that changes in DNA methylation represented by T1D-MVPs must arise very early in the etiological process that leads to overt T1D. These changes involve genes likely associated with the immune response. In addition we have developed a method to identify MVPs in small tissue samples with limited DNA amounts. Our EWAS of T1D represents the first systematic analysis of the temporal origins of disease-associated epigenetic variation for any human complex disease. The development of mini-Medip-Seq will enable us to translate this data into different T1DM-related tissues, even when the amount of tissue is limiting.

Emerging Technologies and Novel Research Avenues

Feasibility of Flow Cytometric Analysis of nPOD Bone Marrow Specimens

Gaia Spinetti[1], Orazio Fortunato[1], and Paolo Madeddu[2]
[1]Laboratory of Diabetological Research, MultiMedica IRCCS, Milan, Italy; [2]Bristol Heart Institute, University of Bristol, Bristol, UK

Purpose: Diabetes-induced dysfunction of bone marrow (BM)-derived stem cells (SCs)/progenitor cells (PCs) may contribute to peripheral cardiovascular complications and microvascular disease. We recently reported the occurrence of microangiopathy in BM of diabetic mice, which causes critical reduction in perfusion, oxidative stress, PC loss through apoptosis and disturbance of endothelial cells (EC)-PCs interaction. We now aim to verify if similar adverse remodelling affects the vascular and haematopoietic components of BM in diabetic patients. Therefore, we tested the feasibility of flow cytometry analysis (FACS) of antigenically-defined cellular subpopulations in human BM samples from nPOD project.

Methods: The effect of diabetes on BM-PCs was assessed analyzing three nPOD iliac crest BM cases: 1) case 6126, not diabetic (ND) male (M), age 25 years, 2) case 6161, Type 1 diabetic (T1D), M, age 19 years, and 3) case 6132, T2D, female, age 52 years. Frozen specimens were thawed following nPOD instructions. FACS analysis was performed to detect distinct cellular subpopulations in the 7AAD-negative fraction (please see Table 1 below for details on the antigenic profile of subpopulations tested).

Summary of Results: Although freezing/thawing procedure resulted in depletion of the total number of cells available for the analysis, the percentage of dead cells was relatively low. FACS analysis confirmed that thawed cells were alive (7AAD negative) in all the three samples tested. We were also able to detect all the cellular subpopulations under investigation and obtained percentages in line with current literature data (please see Table 2 below for FACS results).

Conclusions: We successfully verified a standard operating protocol to thaw nPOD BM specimens in perspective of further cytometric analysis. Samples were adequate to measure the percentages of different populations of BM cells. These preliminary feasibility studies open up the possibility of exploiting human samples from nPOD to investigate the action of diabetes in inducing alterations in BM composition. Moreover, in consideration of the high cell viability, the current method may be exploited to explore BM PC function including the angiogenic properties of early and late EPCs.

(Tables on next page)

Table 1. Definition	Antigenic profile
Hematopoietic PCs	$CD34^{pos}$, $CD133^{pos}$, and c-kit ^{pos}
T-lymphocytes	$CD45^{pos}/CD3^{pos}$
B-lymphocytes	$CD45^{pos}/CD19^{pos}$
Natural Killer cells (NKs)	$CD3^{neg}/CD56^{pos}/CD16^{pos}$
Mesenchymal Cells (MSCs)	$CD73^{pos}/CD105^{pos}/CD90^{pos}/CD34^{neg}/CD45^{neg}$
Endothelial cells (ECs)	$CD45^{neg}/CD31^{pos}/CD144^{pos}$
Early endothelial PCs (eEPCs)	$CD34^{pos}/CD14^{pos}/CD45^{pos}/KDR^{pos}/CXCR4^{pos}$
Late EPCs (lEPCs)	$CD34^{pos}/CD14^{neg}/CD45^{neg}/KDR^{pos}/CXCR4^{pos}$

Table 2. FACS (% of indicated MNC population)	ND	T1D	T2D
$CD34^{pos}$	2.8	0.6	0.9
$CD133^{pos}$	0.2	0.3	0.3
c-kit ^{pos}	2.3	1.6	0.9
T-lymphocytes	17.3	11.2	23.8
B-lymphocytes	8.5	3.2	4.6
NKs	3.3	3.7	8.2
MSCs	0.003	0.03	0.01
ECs	0.03	0.43	0.5
eEPCs	0.04	0.13	0.2
lEPCs	0.02	0.13	0.1

Flow Cytometric Immunophenotyping of nPOD Donors

Christopher Fuhrman, James Thompson, Howard Seay, and Todd Brusko
Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL

Purpose: Cells of both the innate and adaptive arms of the immune system influence the pathogenesis of type 1 diabetes. Certain cell subsets may be lost or phenotypically altered following density gradient centrifugation and the process of cryopreservation (e.g., dendritic cells, macrophages, etc). In an effort to gather and preserve this data for all nPOD users, we set out to develop SOPs to monitor major cellular subsets from freshly isolated peripheral blood and tissues derived from nPOD donors prior to cryopreservation and distribution.

Methods: We have developed polychromatic flow cytometric panels to gather information related to cell frequency, phenotype, and viability (initial and post-thaw) from peripheral whole blood, spleen, pancreatic draining lymph nodes, and non-pancreatic draining lymph nodes. While not comprehensive, these measurements include the analysis of monocytes, plasmacytoid and myeloid dendritic cells, B-lymphocytes, CD8+ T cells, CD4+ conventional and regulatory T cells, NK cells, and iNKT cells. In addition to defining these key populations, we have also added phenotypic markers commonly used to survey MHC class II expression, co-stimulatory ligands, and chemokine receptors.

Summary of Results: Analysis of whole blood cell populations has shown a considerable degree of heterogeneity between nPOD donor samples and normal healthy control peripheral blood samples. Notably, there is a paucity of CD11c+ or CD123+ dendritic cells present in the circulation of nPOD donor subjects. In addition, we have noted tissue-specific alterations in the cytokine profile of Tregs derived from peripheral blood, spleen, and lymph nodes in their capacity to produce IFN-γ and IL-10 upon activation with PMA and ionomycin.

Conclusions: Understanding the viability and functional state of immune cell subsets prior to cryopreservation may have important implications for interpreting nPOD investigator data. Information regarding cell viability and data related to basic immunophenotyping will be provided to nPOD program staff for distribution to program investigators. We expect this data to provide the community of nPOD investigators critical data related to the phenotype and viability of immune cell subsets, and will hopefully facilitate key insights into the etiology of type 1 diabetes.

Development of T1D in Humanized Mice Engrafted with Tissues from Autoimmune Donors

Leonard D. Shultz[1], Michael A. Brehm[2], David Harlan[3], Abdulkadir Omer[3], Sally C. Kent[3], and Dale L. Greiner[2]

[1]The Jackson Laboratory, Bar Harbor, ME; [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA; [3]Department of Medicine, University of Massachusetts Medical School, Worcester, MA

Purpose: Our understanding of type 1 diabetes (T1D) pathogenesis has been advanced greatly by rodent model studies. However, rodents are not humans. Further, current imperfect assays for the analysis of human diabetogenic T cell populations *in vivo* have limited our capabilities to elucidate human T1D pathogenesis, and to test immunotherapies without placing individuals at risk. The availability of spleen, bone marrow, and thymus tissues from T1D and islet autoantibody-positive cadaveric donors as well as the availability of peripheral blood mononuclear cells (PBLs) from living T1D donors provides a unique opportunity to analyze the diabetogenic function of these cell populations following engraftment in newly developed "humanized" mouse models.

Methods: We have developed several novel NOD-scid IL2rnull (NSG) HLA-transgenic humanized models optimized for human hematolymphoid engraftment. These mice support high levels of engraftment with human hematopoietic stem cells (HSC) and mature human lymphoid cells. We have observed that transplanting human spleen cells or PBLs from T1D donors into HLA-class I-matched recipients can induce insulitis, but not overt diabetes. One constraint is the short duration of these experiments due to the development of xeno-GVHD, as the engrafted human T cells respond to murine MHC. Another constraint is the lack of HLA matching between the donor cells and the recipient's HLA class II alleles. To further refine the NSG model, we have recently developed strains of NSG mice that express human MHC (HLA) class I but are deficient in murine MHC class I. We also have developed NSG-HLA-class II transgenic mice that are deficient in murine MHC class II. These NSG mice deficient in murine MHC class I or class II expression display reduced xeno-GVHD responses following human T cell engraftment, permitting longer experimental observation periods.

Summary of Results: We are currently matching the lymphocytes obtained from T1D donors and NSG mouse recipients at both HLA class I and class II alleles in combination with knocking out the mouse MHC class I or class II alleles to determine whether the insulitis will progress to overt diabetes in these recipients. In addition, we have initiated experiments using hematopoietic stem cells from T1D donors engrafted into optimized NSG-HLA transgenic mice matched with the donor's HLA class I and class II alleles.

Conclusions: Based on the induction of insulitis when using HLA Class I matched PBLs from T1D donors, the use of these newly developed mouse strains may permit the recapitulation of human T1D where actions of potential therapies can be examined and manipulated.

Quantum Dot MHC Multimers

Joana R. Ferreira de Abreu and Bart O. Roep
Department of Immunohaematology, Leiden University Medical Center, National Diabetes Expert Center, Leiden, The Netherlands

Purpose: We recently developed a novel strategy allowing simultaneous detection of multiple islet autoreactive CD8 T-cells through specific binding of their T-cell receptor epitope recognition domain. We wish to relate the islet autoreactivity profile of CD8 T-cells in insulitic lesions with that of other regions in the body of this same pancreas donor (pancreas draining and non-draining lymph nodes, peripheral blood and/or spleen), using this novel methodology.

Methods: This strategy involves a combinatorial quantum dot MHC multimer nanotechnique to simultaneously monitor the presence of HLA-A2 restricted CD8+ T-cells against a wide range of candidate islet autoantigens (insulin, pre-pro-insulin, IA-2, GAD65, IGRP and ppIAPP) that has been validated in recent onset diabetes patients, their siblings, healthy controls and islet cell transplantation recipients. These novel HLA multimer reagents have been scrutinized for their specificity, peptide epitope purity and valency. Using this kit, islet autoreactive CD8+ T-cells recognizing a range of islet autoantigens were shown to be preferentially detectable in recent onset patients, but rarely in healthy controls. Applying this methodology to samples of islet cell transplantation recipients allowed detection of changes of autoreactive T-cell frequencies against multiple islet cell derived epitopes that was associated with disease activity and correlated with clinical outcome. Our novel approach allows simultaneous detection of CD8+ T-cells reactive to multiple HLA-A2-restricted beta cell epitopes requiring limited amounts of blood, without a need for in vitro culture (in contrast the current methods for detection of islet autoreactive T-cells), that is applicable on stored blood samples.

Summary of Results: Using the very same HLA class I tetramers and islet epitopes, we are currently comparing insulitic T-cell specificities with that in other tissues. Since we only need very small aliquots of cryopreserved PBMC, PDLN or spleen cells, our request should supplement other current activities in cellular islet autoimmunity in nPOD. In the context of current nPOD activities at the LIAI (Dr. Von Herrath) we have investigated T-cell autoreactivity in situ in insulitic lesions using the same source of HLA monomers. Preliminary data suggest that mesenteric lymph nodes drain the pancreas as indicated by increased frequencies of T cells to GAD65, in contrast to mice, and therefore seem to disqualify as `control' tissue.

Conclusions: Our technology delivers in nPOD T cell specifities detectable in circulation and lymph nodes can be found in insulitis. We currently define the frequency and specificity of islet autoreactive CD8 T-cells in pancreas draining lymph nodes, control lymph nodes, spleen and/or peripheral blood of larger series of HLA-A2 positive diabetic and prediabetic organ donors and compare islet autoreactivity in periphery with that in insulitic lesions.

The Role of IA-2 Extracellular Domain in Type 1 Diabetes Progression

Michael Morran[1], Les Satin[2], Roberto Townes[1], Joshua Pohlman[3], and Massimo Pietropaolo[1]
[1]MEND, University of Michigan, Brehm Center for Diabetes Research, Ann Arbor, MI; [2]Pharmacology, University of Michigan, Brehm Center for Diabetes Research, Ann Arbor, MI; [3]School of Medicine, University of North Dakota, Grand Forks, ND

Purpose: The tyrosine phosphatase-like protein (IA-2) is a major autoantigen associated with the progression and detection of T1D. We identified a new candidate biomarker within the extracellular domain of IA-2 (IA-2ec) in humans, which leads to rapid acceleration of T1D onset compared to conventional IA-2 biomarkers of T1D. Our objective is to identify autoantibody binding domains specific for IA-2ec, in order to determine their pathophysiological impact on cell function. Our central hypothesis is that autoantibodies present during the progression of T1D are pathogenic and cause distinct physiological alterations in cellular metabolism.

Methods: We synthesized and assayed a new bio-marker construct against the human IA-2ec, amino acids 26-577, through standard serum autoantobody screening via radio-immunoassay (RIA). Competitive bindings studies utilizing IA-2ec and standard bio-markers associated with the progression of T1D were also carried out to fully characterize IA-2ec recognition. Human SH-SY5Y neuroblastoma cells were differentiated and treated with poly-clonal (Santa Cruz) IA-2ec antibodies, wherein they were assayed to determine possible physiological effects on calcium channel activity and calcium mobilization upon antibody treatment. Lastly, nPOD subjects previously identified as reactive for conventional IA-2 or IA-2 derived bio-markers were screened for the presence of IA-2ec via RIA.

Summary of Results: We developed a new bio-marker construct against the human IA-2ec, which successfully identifies IA-2ec positive individuals through standard serum RIA. This response is associated with a high risk of progression toward T1D. Competitive binding experiments further support the presence of autoantibody responses directed toward the extracellular domain of IA-2. Upon treatment with poly-clonal IA-2ec antibodies, differentiated SH-SY5Y cells displayed significant increases in mobilized calcium and calcium channel activity compared to isotype treated controls. In addition, a small set of nPOD subjects previously identified as being positive for conventional IA-2 based biomarkers, were able to be identified as positive for IA-2ec autoantiboides.

Conclusions: Our data shows that the extracellualr domain of IA-2 is a novel antigenic determinant associated with rapid progression to T1D. Furthermore, antibodies reactive toward IA-2ec have the ability to cause distinct physiological alterations in calcium channel signaling and the mobilization of calcium. Future studies utilizing IA-2ec postive nPOD subjects will be carried out to elucidate IA-2ec autoantibody binding sequences to afford the production of monoclonal antibodies and Fab fragments specific for IA-2ec recognition.

In-situ Reduction Coupled with Imaging Mass Spectrometry as a Novel Method for T1D Biomarker Identification in nPOD Tissue Samples

Shamina M. Green-Mitchell[1], Lisa H. Cazares[2], Wojciech Grzesik[1], Margaret A. Morris[1], Julius O. Nyalwidhe[2], and Jerry L. Nadler[1]
[1]Internal Medicine, Eastern Virginia Medical School, Norfolk, VA; [2]Leroy T. Canoles Cancer Research Center, Eastern Virginia Medical School, Norfolk, VA

Purpose: There is an unmet medical need to identify novel biomarkers of type 1 diabetes (T1D). In T1D insulin producing cells are the selective target for inflammatory autoimmune destruction. Our long-term goal is to characterize differentially expressed proteins in human pancreatic tissues using matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI). The ability to track relative insulin expression during MALDI-MSI allows for verification of β-cell status and novel pathways leading to beta cell damage though in-situ MS-MS has been used in the identification of molecules directly from tissues, there are technical limits on the identification of biomolecules with molecular masses ≥ 4000 Da. We have used in-situ reduction of disulphide bonds in the proteins of pancreatic tissue sections to generate optimum sized components that are amenable to direct MS/MS. Presently, there is no known cure for T1D and diabetics endure the invasive conventional treatment of insulin replacement therapy. Here, we apply MALDI-IMS after on- tissue reduction to mine the proteome of the pancreatic islet of Langerhans as a means of identifying key players involved in insulitis.

Methods: Human Diabetic and non-diabetic pancreatic section donated from Network for Pancreatic Organ Donors with Diabetes nPOD were placed in OCT and snap frozen in 2-methylbutane submerged in liquid nitrogen. Tissue slices were cut 7µm thick in a -20°C cryostat and mounted on glass slide for hematoxylin and eosin staining. A sequential slice was cut and mounded onto an indium coated mass spectrometry slide for MALDI-IMS. The tissue was then washed and fixed in ethanol and left to dry in a desiccator until it was homogeneously sprayed with matrix in an automated sprayer (Bruker Daltonics). The sprayed tissue was subjected to MALDI-IMS. Using a pre-clinical model in which a target gene was deleted in the nod mouse, this tool was able to detect unique proteins of interest that could distinguish between the diabetic NOD and protected non-diabetic NOD-Alox15null.mice. In the human tissue samples, we have used in-situ reduction of disulphide bonds in the proteins of pancreatic tissue sections to generate optimum sized components (≥ 4000 Da) that are amenable to direct MS/MS.

Summary of Results: Mass spectrometry data showed that we can clearly differentiate between the pancreases of a NOD versus NOD-Alox15null using IMS to identify the islets and Langerhans and potential novel biomarkers that are specific to each group. In human tissue, we succeeded in the identification of mature insulin, a 5.8 kDa multidomain protein comprised of smaller A and B chains. Reduction generated the individual chains and MS/MS analysis effectively identified the B chain with an m/z of 3430.664. The MALDI-MSI image of the 5812.85 insulin peak before reduction and the 3430.664 peak after reduction both co-localized with the healthy pancreatic islets.

Conclusions: This study showed that insulin, a relatively large molecule can be easily identified in human pancreatic sections via MALDI-IMS. This approach is now being used to determine differential protein expression between a human diabetic and non-diabetic pancreas. We expect to identify candidates to be useful in the identification of therapeutic molecular targets in β-cells.

In Vitro Evaluation of Non-Specific Binding of the Candidate Beta Cell Mass PET Probe (+) 18F-FP-DTBZ

Antonella Maffei and Paul Harris
Division of Endocrinology, Department of Medicine, Columbia University Medical College, New York, NY

Purpose: The vesicular monoamine transporter Type 2 (VMAT2) is a candidate beta cell mass (BCM) marker that can be evaluated non-invasively by PET using the radioligand (+) 18F-FP-DTBZ. We evaluated the utility of this BCM marker and radioligand in a cross sectional studies of healthy human volunteers and subjects with long standing type 1 diabetes, predicted to have little or no beta cell mass based on metabolic measurements. Using Binding Potential (BP) as the PET outcome measure, our in progress studies reveal that healthy controls (n=9) have an average BP = 2.15 compared to an average BP = 1.37 (63% of controls) found in T1D subjects (n=5). The significant PET signal in the T1D population is unexpected as histological evaluation of the amounts of VMAT2 in healthy controls and T1D pancreata correlate closely with insulin staining. One explanation for binding of the PET probe in T1D pancreata is that (+) 18F-FP-DTBZ binds not only specialty and displaceably to VMAT2 but non-specifically to pancreas membranes as well.

Methods: We evaluated non-specific binding of (+) 18F-FP-DTBZ in membrane preparations of human pancreata in vitro using a receptor binding assay as an estimate of the in vivo levels of non specific probe binding potentially observed in our clinical PET studies. Snap frozen pancreas tissue was received from nPOD and total pancreas membranes prepared in the presence of a protease inhibitor cocktail. Membrane protein concentrations were measured by the bicinchoninic acid method for normalization of the protein content of membranes samples obtained from different individuals. Aliquots of membranes were diluted in cold assay buffer [0.3 m sucrose, 25 mm HEPES (pH 7.5), and 5 mm MgCl2, containing a protease inhibitor mixture (100 µm PMSF, 100 µm benzamidine, 20 µg/ml leupeptin, and 10 µg/ml soybean trypsin inhibitor; final concentrations)]. 18F-FP-DTBZ (Specific Activity 900-1100 Ci/mmol) was added to 25 nM in the presence or absence of a 1000 molar excess of cold tetrabenazine in triplicate determinations. The reaction was terminated by the addition cold assay buffer and washing filters on a vacuum manifold. Radioactivity trapped in filters was counted in a gamma counter.

Summary of Results: The total and displaceable binding was measured in membrane samples obtained from eight individuals including one subject with long term T1D (<0.05 ng/ml c-peptide) and one subject with T2DM
(0.85 ng/ml c-peptide). The specific displaceable binding (total minus nondisplaceable binding) in healthy subjects with normal or near normal c-peptide averaged 40% of the total (range 22-56%). As predicted membranes prepared from the long term T1D pancreas showed no displaceable probe binding.

Conclusions: Our preliminary data suggest that there is non-displaceable non specific binding of (+) 18F-FP-DTBZ to pancreas membranes in vitro. Extrapolating the magnitude of the in vitro non specific binding of 18F-FP-DTBZ to our in vivo PET measurements of VMAT2 and BCM, suggests that the true in vivo measured BP (corrected for non-specific binding) for normal controls will be closer to 1 and that of individuals with no detectable BCM will be closer to 0 as predicted by metabolic measures. Our in vitro findings are currently being validated in an in vivo study using a complementary PET technique applying the non VMAT2 binding (-) stereoisomer of (+) 18F-FP-DTBZ.

Pancreas and Beta Cell Biology

The Pancreatic Duct Gland Compartment in Non-Diabetic and Type 1 Diabetic Subjects

Alexandra E. Butler[1], Belinda Gier[1], Mark Atkinson[2], and Peter C. Butler[1]
[1]Department of Medicine/Endocrinology, University of California – Los Angeles; [2]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL

Purpose: Pancreatic duct glands (PDGs) are coiled blind ending glandular structures that arise from pancreatic ducts and express stem cell markers such as HES-1. The PDG compartment has recently been appreciated as an adult stem cell niche in pancreas that undergoes proliferation and expansion in response to acute exocrine pancreatic injury. As yet there is no data as to whether the PDG compartment gives rise to pancreatic beta cells. We posed the following questions. First, does the PDG compartment contain beta cells in humans with type 1 diabetes (T1DM)? Second, is there an increase in proliferation of the PDG compartment in T1DM?

Methods: Pancreas sections were made available from the nPOD consortium from the head, body and tail of pancreas and stained with Alcian Blue to identify PDGs, insulin immunohistochemistry to identify beta cells and Ki67 for replication.

Summary of Results: Beta cells were identified in PDGs in T1DM and non diabetic controls implying that PDGs may be a stem cell niche for beta cell formation in adult humans. However the number of beta cells in PDGs is decreased by approximately 50% in T1DM implying decreased cell formation and/or increased beta cell destruction. To approach this we quantified PDG cell replication which is increased ~4 fold in T1DM.

Conclusions: Taken together these preliminary findings imply that the PDG compartment may be a stem cell niche for beta cell formation in adult humans with attempted increased beta cell regeneration overcome by ongoing beta cell destruction. Cell lineage studies of the PDG compartment are required to establish that this compartment is a potential beta cell source and by what means cell fate is regulated from this stem cell niche.

Formation of a Baseline Pancreatic β-cell Mass in Humans is Completed Early in Life

Brigid Gregg[1], Damien Demozay[1], Patrick C. Moore[1], Mei Li[2], Aliya Husain[2], and Christopher J. Rhodes[1]
[1]The Kovler Diabetes Center, Department of Medicine, University of Chicago, Chicago, IL; [2]Department of Pathology, University of Chicago, Chicago, IL

Purpose: The baseline complement of pancreatic islet beta cells in an individual can be pivotal in the pathogenesis of diabetes. In type 1 diabetes, it may predict the extent of an autoimmune attack. In type 2 diabetes, it can dictate whether beta cell mass can compensate, or not, in the face of an increased metabolic load/insulin resistance. However, there have been a limited number of studies in humans and it is unclear how, and when, a baseline beta cell mass forms. Moreover, since a fully functional beta cell requires interaction with other islet hormone producing cell types and associated cells (e.g., neuronal cells), the formation of normal human beta cell mass relative to other pancreatic cell types is important. Here we have examined the formation of normal human beta cell mass relative to alpha-, delta-, and pancreatic neuronal cells from late development, neonatal, childhood, adolescent through to adulthood.

Methods: Pancreatic sections (10 µm thick) were analyzed by immunofluorescent staining and confocal microscopy for the formation of alpha-(glucagon), beta-(insulin), delta-(somatostatin) cells relative to each other. Ductal (CK-19) and beta cell proliferation was examined by Ki67 staining and apoptosis by activated caspase-3. The neogenesis of beta cells (insulin+ cell emerging from or associated to pancreatic ductal cells) was also assessed. The formation neurons proximal to pancreatic islet beta cells was examined using TUJ1/MAP2 as a neuronal specific marker. Each section was quantified by morphometric counting analysis.

Summary of Results: The baseline pancreatic beta cell mass was formed early in neonatal human life. This was a result of beta cell neogenesis mostly occurring developmentally and then a burst of neonatal beta cell proliferation, which doubled the beta cell population by age 2 years. Thereafter, the beta cell population stayed relatively constant with very low levels or neogenesis, proliferation and apoptosis. The pancreatic alpha and delta-cell populations appeared to be set at birth. The beta cell:alpha-cell ratio approximately doubled in the first year of life – reflective of the burst of beta cell proliferation rather that change in alpha-cell numbers. The beta cell:delta-cell ratio changed more dramatically (~8-fold) through childhood from birth reflecting a marked loss of delta-cells in the first 10 years of life. A neonatal burst of ductal cell proliferation was also observed that followed the burst of beta cell proliferation, and was more likely associated with expansion of pancreatic exocrine cells as formation of pancreatic islets occurred. Finally, it was found that ≥90% of detectable pancreatic neurons first associated with pancreatic endocrine islet beta cells developmentally, and this tight association was retained throughout adulthood. Few pancreatic neurons associated with exocrine tissue.

Conclusions: The baseline complement of human pancreatic islet beta cells is set early, by ~2 years old. There is a tight association of neurons and pancreatic alpha-cells to islet beta cells, but islet delta-cells are lost during childhood. This study has implications to intra-uterine fetal programming having influence on the formation of baseline beta cell mass, and, if dysfunctional, could contribute to the pathogenesis of diabetes later in life.

β-cells Persist in Some T1DM Pancreata without Attempted Evidence of β-cell Turnover nor Insulin-Glucagon Co-expression

Daniel R. Jacobson[1], Mathew M. Rankin[1], Changhong Li[1], Alisa B. Schiffman[1], and Jake A. Kushner[2]
[1]Division of Endocrinology and Diabetes, Children's Hospital of Philadelphia, UPenn SOM, Philadelphia, PA; [2]Section of Pediatric Diabetes and Endocrinology, Baylor College of Medicine, Houston, TX

Purpose: Regeneration of ß-cell function in T1DM is a fundamental research goal that requires improved knowledge of the lineage mechanisms of ß-cell growth and regeneration. ß-cell function is detectable in some T1DM patients, even some with longstanding disease. However, the developmental basis of persistent ß-cell function remains an utter mystery. Despite decades of research in animal models ranging from mouse to dog to pig, there is little quantitative data of ß-cell lifecycle in normal pancreata, in part due to the extremely low rates of endogenous ß-cell turnover in the basal state and the lack of robust high-throughput imaging methodologies. Importantly, ß-cell turnover has never been accurately measured in T1DM pancreata. We sought to establish such rates in both T1DM patients and controls across age groups with robust high-throughput methods.

Methods: We performed immunofluorescent staining of JDRF nPOD pancreata, quantifying total islet endocrine cells (Synaptophysin), ß-cells (insulin), and alpha cells (glucagon). To determine replication, we stained for Ki67. To measure death we used the TUNEL staining method combined with hormone specific markers. We used an automated X-Y stage with a motorized fluorescent microscope to obtain images from all visible islets from each section, which resulted in huge image files from each case comprising tens of thousands of individual nuclei. We then employed a proprietary image-processing algorithm written for the Perkin Elmer Volocity platform to analyze the images. We counted an average of 43,000 nuclei per sample per staining group across 41 samples (20 T1DMs and 21 controls). In total our findings summarize hormone expression data from upwards of 5.5 million individual nuclei, achieving unprecedented levels of quantitative rigor.

Summary of Results: Residual insulin producing ß-cells were detected in some (but not all) T1DM cases. Several T1DM cases had substantial numbers of ß-cells. However, T1DM pancreata with persistent ß-cells did not exhibit increased ß-cell or islet endocrine cell replication when compared to controls, or to T1DM cases without ß-cells,. Similarly, T1DM pancreata with persistent ß-cells had equivalent rates of ß-cell or islet endocrine cell death compared to controls, or to T1DM cases without ß-cells. As expected from previously published rodent studies, ß-cell and islet endocrine cell replication decreased with age in both control and T1DM pancreata. ß-cell death was infrequent in both control and T1DM pancreata, with no age specific associations. Islet endocrine cells that co-expressed insulin and glucagon were not detected in control pancreata, nor in T1DM pancreata.

Conclusions: A few ß-cells often persist in T1DM pancreata. However, there is no evidence of ß-cell regeneration in T1DM pancreata, except within a single unusual case (#6052). Human islet endocrine cell replication decreases with age. Human postnatal pancreatic ß-cells do not co-express insulin and glucagon, even in T1DM pancreata. Thus, longstanding ß-cell function in T1DM patients appears to be largely due to ß-cells that simply persist, without any evidence of attempted ß-cell regeneration nor trans-differentiation from other endocrine cell types. Future ß-cell regeneration therapies must overcome very low endogenous rates of ß-cell turnover in order to achieve substantial islet endocrine cell mass in T1DM patients.

Cytoplasmic-Nuclear Trafficking of G1/S Cell Cycle Molecules: A Novel and Critical Regulatory Mechanism Controlling Adult Human Beta Cell Replication

Nathalie Fiaschi-Taesch, Fatimah Salim, Jeffrey Kleinberger, Ronnie Troxell, Amy Cox, Harish Shrinivas, Don Scott, Karen Takane, and Andrew Stewart
Department of Medicine, Division of Endocrinology, University of Pittsburgh, Pittsburgh, PA

Purpose: Adult human ß cells are resistant to attempts at inducing proliferation. Proliferation is controlled by a family of ~30 G1/S molecules (8 E2Fs, 3 pRb members, 4 INK4s, 3 KIP/CIPs, 4 cdks and 7 cyclins, and others), and several (eg., cdks 2, 4, 6, cyclins D1-3, E) can induce adult human ß cell replication. We now have an islet G1/S molecule roadmap, yet since it was derived from immunoblots of whole human islets, it does not document which, if any, of the G1/S molecules are actually present in the human ß cell. Here, our initial goals were to define which G1/S molecules are present in the human ß cell, and to develop an immunohistochemical (IHC) human ß cell G1/S molecule "atlas". We assumed these molecules would reside in the nuclear compartment.

Methods: Using IHC in dispersed human islets, all 30 G1/S molecules, except cyclin D2, were observed in the human ß cell. Surprisingly, however, all were cytoplasmic, and absent from the nucleus, with only three exceptions: pRb, p21 and p57. This was independently evaluated for each G1/S molecule using subcellular fractionation of human islets, which confirmed that all but pRb, p21 and p57 are cytoplasmic, not nuclear, proteins. Most importantly and to rule out any artifact due to islets isolation, we determined the expression and localization of some of these G1/S molecules in intact human pancreas sections. While pRb and p57 were confirmed to be nuclear, cdk6, p18 and p107 were found cytoplasmic in human beta cells.

Summary of Results: We asked whether induction of proliferation might alter the subcellular localization of the 30 G1/S molecules in the ß cell. Adenoviral overexpression of cyclin D3 and cdk6 led to brisk increases (30-50x; from 0.3%, to 10-15%) in adult human ß cell proliferation (BrdU, Ki67). The nuclear presence of cdk6 and cyclin D3 increased dramatically (basal 0%, stimulated ~40%). In addition, p16, p21 and p27 also migrated to the nucleus (basal ~10%-7%-2% respectively, stimulated 20%-35%-15% respectively), whereas other G1/S members remained cytoplasmic. p57 was less frequent in the nucleus (basal ~40%, stimulated 25%). Interestingly, nuclear trafficking of cdk6 occurred early (within 24h) and remained constant for 72-96h, whereas cyclin D3 nuclear entry appeared within 24h, peaked by 48h, and declined precipitously by 72h. Critically, proliferation occurred predominantly in cells that were positive for nuclear cyclin D3 and/or cdk6, and negative for nuclear 16, p21, p27 or p57.

Conclusions: This study provides the first comprehensive human ß cell G1/S "atlas". It shows that all G1/S molecules except for cyclin D2 reside in the human ß cell, and that cell cycle control molecules, widely assumed to be nuclear, are in fact cytoplasmic, but can traffic to the nucleus in association with activation.

Residual Beta Cells in Autoimmune Type 1 Diabetes Mellitus

Roberto Gianani[1], Martha Campbell-Thompson[2], Matthew Maulis[1], Clive Wasserfall[2], Mark Atkinson[2], Alberto Pugliese[3], and Susan Bonner-Weir[4]
[1]Barbara Davis Center for Childhood Diabetes, University of Colorado – Denver, Aurora, CO;
[2]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL;
[3]Diabetes Research Institute, University of Miami Medical School, Miami, FL; [4]Joslin Diabetes Center, Harvard Medical School, Boston, MA

Purpose: Autoimmune Type 1 diabetes is a disease characterized by chronic beta cell loss which continues after onset and positivity for anti-islet autoantibodies. Given the insulin dependence typical of patients with autoimmune diabetes, it is generally believed that there is little or no residual beta cell mass in patients with long standing disease. Previous reports have however shown that amongst patients with childhood diabetes (most of whom presumably have autoimmune diabetes) a subset of individuals maintains residual beta cell mass despite long term follow-up. The interpretation of this data is complicated by the heterogeneity of Type 1 diabetes with two major pattern of pathology identified in childhood diabetes characterized by the presence or absence of pseudoatrophic islets (i.e. islets without insulin positive cells).

Methods: In order to better assess the prevalence of residual beta cells in patients with autoimmune Type1 diabetes, we utilized samples from the JDRF sponsored nPOD (network of pancreatic organ donors) initiative that were characterized for islet cell autoantibodies as determined by radioimmunoassay for the islet autoantigens GAD65, ICA512 (IA-2) and Znt8. To minimize the possible effect of diabetes heterogeneity in the assessment of beta cell mass preservation in patients with autoimmunity, we defined as “autoimmune diabetes” only clinically defined Type1 diabetes accompanied by positivity for at least one islet autoantibody. When this definition was applied to subjects with long standing diabetes (at least one year of disease duration) screened through the nPOD program, we identified 17 islet antibodies positive donors with mean disease duration of 10.18 years, average age at death of 23.42 years and average age of onset of 13.25 years. In this group, 29% (5/17) of subjects had residual insulin positive cells in the pancreas.

Summary of Results: Quantification of this beta cell mass by morphometric analysis revealed that the mean beta cell mass of subjects whose pancreas contained residual insulin positive beta cells was less than 1% of the mean value in normal controls. These islets show a decreased ratio of beta to non-beta islet cells. This suggests that, albeit not destroyed, they were subject to autoimmune attack. This was also confirmed by the findings of insulitis in some of these cases. The pancreata of all individuals with Type 1 autoimmune diabetes contained pseudoatrophic islets (regardless of the presence or absence of residual beta cells) (Pattern A beta cell loss).

Conclusions: In conclusion, these data show that autoimmune Type 1 diabetes is characterized by pattern A beta cell loss and a significant proportion of individuals with Type 1 autoimmune diabetes maintains residual beta cells.

Case Processing

nPOD Case Processing

Emily Montgomery, Irina Kusmartseva, Maria Martino, Li Zhang, Lynda Schneider, Robin Foss, Gerald Phipps, Kerwin Kolheffer, and Martha Campbell-Thompson
Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL

Purpose: To provide a diverse set of high quality human tissues for Type 1 Diabetes research to approved investigators in order to enhance their ability to study the disease as it occurs in humans.

Methods: Through partnerships with Organ Procurement Organizations, nPOD is able to regularly obtain pancreas, spleen, duodenum, lymph nodes, blood, and serum. On occasion, thymus, skin, bone marrow and vertebral bodies are also received. In order to consistently provide investigators with high quality tissues, each case is processed following a standard operating procedure. The sample quality is optimized and maintained through quality control measurements. Each tissue is processed into several different sample types in order to best fit the needs of nPOD investigators. The sample types are as follows: paraffin blocks, fresh frozen blocks (OCT), snap frozen vials, snap frozen vials with RNALater, DNA isolated from received tissues, cells isolated from spleen, pancreatic lymph nodes, non-pancreatic lymph nodes, thymus, blood marrow aspirate, and mononuclear cells from peripheral blood.

Summary of Results: nPOD is providing rare and previously difficult to obtain human tissues to investigators. nPOD is constantly expanding and to date more than 170 cases have been processed with over 550 distributions to investigators. This growth has allowed researchers to receive a greater variety of human tissue samples providing them the opportunity to increase the depth and scope of their research.

Conclusions: nPOD has consistently provided a variety of high quality human tissues related to Type 1 Diabetes to approved investigators and has continued to grow according to the needs of affiliated investigators.