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Investigation and Characterisation of T-Lymphocytes in Chronic Pancreatitis

by

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ABSTRACT

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INVESTIGATION AND CHARACTERISATION OF T-LYMPHOCYTES IN CHRONIC PANCREATITIS

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It has been suggested that T-lymphocytes play a role in the pathogenesis of chronic pancreatitis (CP), but little is known about the composition of T-cell subsets in this disease. I therefore aimed to characterise T-lymphocytes and in particular T-helper (Th), T-regulatory (Treg) and mucosal-associated invariant T- (MAIT) lymphocytes in CP tissue and peripheral blood.

I first developed a reproducible method to extract infiltrating mononuclear cells to allow their further examination. Mononuclear cells isolated from the pancreatic tissue and blood of patients with CP and controls were then examined using flow cytometry. Pancreatic tissue lymphocytes were also assessed using immunohistochemistry.

The following results were obtained: Pancreatic tissue from patients with CP contained a significant infiltrate of CD3+ T-cells comprised of equal proportions of CD4+ and CD8+ cells. There was a proportional reduction in CD4+ T-cells in pancreatic tissue when it was compared to blood in CP patients, but no difference in CD8 or double negative (DN) T-cells was detected. The peripheral blood of CP patients trended towards a lower proportion of CD4+ and higher a proportion of CD8+ T-cells compared to controls, with an associated trend of a lower CD4:CD8 ratio. A trend towards a lower proportion of CD8+ Integrin α4β7+ cells was also noted suggesting their accumulation in inflamed pancreatic tissue.

This work is the first to demonstrate a significant increase in the number of Th1, Th2, Th17 and Treg cells in the peripheral blood of CP patients compared to controls. Furthermore patients who consume excess alcohol have significantly more Th1 cells than non-drinkers. Pancreas infiltrating CD4+ T-helper cells are predominantly Th1 and Th17 cells, with few or no Th2 or Treg cells identified. MAIT cells were detected in CP but not normal pancreatic tissue and DN MAIT cells were found to be enriched in CP tissue compared to blood.

Interestingly there is no polarisation of the peripheral blood T-helper cell response in CP towards either a Th1 or Th2 phenotype. It appears therefore that the blood of CP patients is primed to respond non-specifically to inflammatory stimuli. Intriguingly patients who consume excess alcohol have more peripheral blood Th1 cells. This may be explained by the fact that alcohol increases gut permeability causing high circulating levels of lipopolysaccharide which is known to generate Th1 cell responses.

The identification of MAIT cells in CP tissue is novel and the enrichment of DN MAIT cells in the tissue of CP patients compared to blood suggests a specific role in local immune responses. These combined features support the notion that the pancreatic inflammatory T-cell infiltrate contributes to the pathogenesis of CP.

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DECLARATION OF AUTHORSHIP

I, James Aubrey Jupp

declare that the thesis entitled

Investigation and Characterisation of T-Lymphocytes in Chronic Pancreatitis

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as poster and oral presentations (See Appendix)

Signed:

Date: 01/07/2012

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This work is dedicated to Becky, Thomas and Oliver.

ABBREVIATIONS

α-SMA	α-Smooth Muscle Actin
AEC	3-Amino-9-Ethylcarbazole
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine Dye 7
CCR	Chemokine (CC motif) Receptor
CD	Cluster of Differentiation
CP	Chronic Pancreatitis
CpG	Cytosine-guanosine Dinucleotides
CT	Computed Tomography
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-associated Antigen-4
DBTC	Dibutylin Dichloride
DM	Diabetes Mellitus
DMSO	Dimethyl Sulfoxide
DN	Double Negative (CD4- CD8- lymphocyte)
ECM	Extra-cellular Matrix
ERCP	Endoscopic Retrograde Cholangio-Pancreatography
EUS	Endoscopic Ultrasound
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead Box P3
GALT	Gut-Associated Lymphoid Tissue
GITR	Glucocorticoid-induced Tumour necrosis factor Receptor family-related gene

GMA	Glycol Methacrylate Resin
GPCR	G-protein Coupled Receptors
HBSS	Hanks Balanced Salt Solution
HLA	Human Leukocyte Antigen
HSC	Hepatic Stellate Cell
IEL	Intraepithelial Lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant Natural Killer T (cells)
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule - 1
MAIT	Mucosal-Associated Invariant T (cells)
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
MR-1	MHC-Related molecule 1
NAC	N-Acetyl Cysteine
NF-κB	Nuclear Factor-κB
NOD	Non-Obese Diabetic
PAC	Pancreatic Adenocarcinoma
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine Dye 7
PE-TR	Phycoerythrin-Texas Red

PerCP-Cy5.5	Peridinin Chlorophyll Protein-Cyanine Dye 5.5
PHA	Phytohaemagglutinin
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PSC	Pancreatic Stellate Cell
ROS	Reactive Oxygen Species
SCID	Severe Combined Immunodeficiency
Tc	T cytotoxic (lymphocyte)
Th	T helper (lymphocyte)
Treg	T regulatory (lymphocyte)
TBE	Tris Borate EDTA
TBS	Tris Buffered Saline
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor Beta
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
WBK	Wistar Bonn/Kobori rat

Chapter 1: Introduction

Chronic pancreatitis (CP) is a progressive inflammatory disease characterised by destruction of acinar, ductal and islet cells, and replacement by fibrous tissue, resulting in exocrine and endocrine pancreatic insufficiency. It is a debilitating illness which causes severe chronic abdominal pain, malnutrition and latterly diabetes, resulting in significant morbidity and mortality. Patients describe a poor quality of life and life expectancy is also reduced; 55% of patients die within 20 years of diagnosis.[1-3] Current therapy is limited to enzyme replacement, analgesics and surgery, as no treatment is available that can alter the natural history of the disease. A few recent studies have suggested a possible role of the immune system, and in particular T-lymphocytes, in the pathogenesis of CP, however, more precise details of the infiltrating immune cell composition are not known. A better understanding of these immunological aspects of CP may therefore provide potential for the research and development of new therapeutic interventions.

A HISTORICAL PERSPECTIVE OF THE PANCREAS

Although the Babylonians had previously described the pancreas as a "*finger of liver"*, [4] it was the ancient Greeks that first identified it as a distinct organ. It was regarded as an unusual organ as it contained no cartilage or bone, which led Rufus of Ephesus (c. 100 AD) to name it pancreas (Greek *pan*: all and *kreas*: flesh). Several centuries later the great anatomist Andreas Vesalius (1514 – 1564) referred to the *"glandulous organ or kannelly body of substance growing in the neather pannicle of the caule (omentum)"* in his Opus, De humani corporis fabrica (On the workings of the human body). He, like many in his footsteps, incorrectly assumed its function, considering it to serve as a protective cushion for the stomach.[5]

The formal study of pancreatic physiology began with Franciscus Sylvius and his student Regnier de Graaf (1641 – 1673) who developed a means of collecting pancreatic juice (*succus pancreaticus)* from dogs using feather quills to create a pancreatic fistula. They concluded that pancreatic juice effervesced with bile to allow digestion of food, however they also incorrectly believed pancreatic juice was acidic, a fact disproved by Johann Bonn (1640 – 1719). Johann Brunner (1653 – 1727) at a similar time studied pancreatectomised dogs that he noted developed polydipsia and polyuria, however he failed to identify the presence of sugar in the urine.[5] It was not until 1788 that the correlation between pancreatic disease and diabetes was made by

Thomas Cawley.[6] Detailed embryology of the pancreas was provided in 1806 by Johann Friedrick Meckel, the Younger. In the meantime the term *abdominal salivary gland* had been popularised and the proximity of the bile duct and the pancreatic duct had led Albrecht von Haller (1708 – 1777) to conclude that pancreatic juice and bile interacted in the process of digestion.

In the mid-19th century Claude Bernard (1813 – 1878) unified the concepts of pancreatic digestion by demonstrating that gastric digestion *"is only a preparation act"* and that pancreatic juice emulsified fatty food and converted starch into sugar.[7] The pancreatic enzymes trypsin and lipase were then identified a few decades later by Willy Kuhne (1837 – 1900) and Alexander Marcet (1770 – 1882) respectively. Paul Langerhans (1847 – 1888) subsequently elucidated the differences between pancreatic exocrine and endocrine tissue, followed by Ivan Pavlov (1849 – 1936) who described the regulation of pancreatic secretion by the vagus nerve. The observation by Chepovalnikov in 1899 that pancreatic juice exerted a powerful action on *proteids* only after contact with either the duodenal wall or extracts from it led to the discovery of enterokinase. Finally works by William Bayliss (1860-1924) and Ernest Starling (1866-1927) introduced the concepts of nervous and hormonal regulation of pancreatic exocrine secretion.[5]

PANCREATIC STRUCTURE AND FUNCTION

Macroscopic Structure

The pancreas is a soft, greyish-pink retroperitoneal organ, situated on the posterior wall of the abdomen. It is approximately 15cm long and consists of a head, neck, body and tail. The head of the pancreas lies within the concavity of the duodenum and is anterior to the aorta and vena cava. The neck is a constricted portion of the pancreas which connects the head to the body, which runs upwards and to the left, across the midline to the tail which is in contact with the hilum of the spleen. The pancreas' blood supply is derived from the splenic and pancreaticoduodenal arteries. Venous blood drains directly into the superior mesenteric and splenic veins, which form the portal vein immediately posterior to the pancreatic head. Lymphatic drainage is directed into retropancreatic, coeliac and superior mesenteric nodes. The main pancreatic duct begins in the tail and runs the length of the gland. It is fed by numerous smaller ducts which channel pancreatic secretions from within lobules. Within the head of the pancreas the main pancreatic duct is joined by the common bile duct, and together they open into the duodenum through the major duodenal papilla, via the

hepatopancreatic ampulla. An accessory duct, when present, drains into the duodenum a short distance above the major duodenal papilla (See Figure 1).[8]

Figure 1: Macroscopic and Microscopic Anatomy of the Pancreas

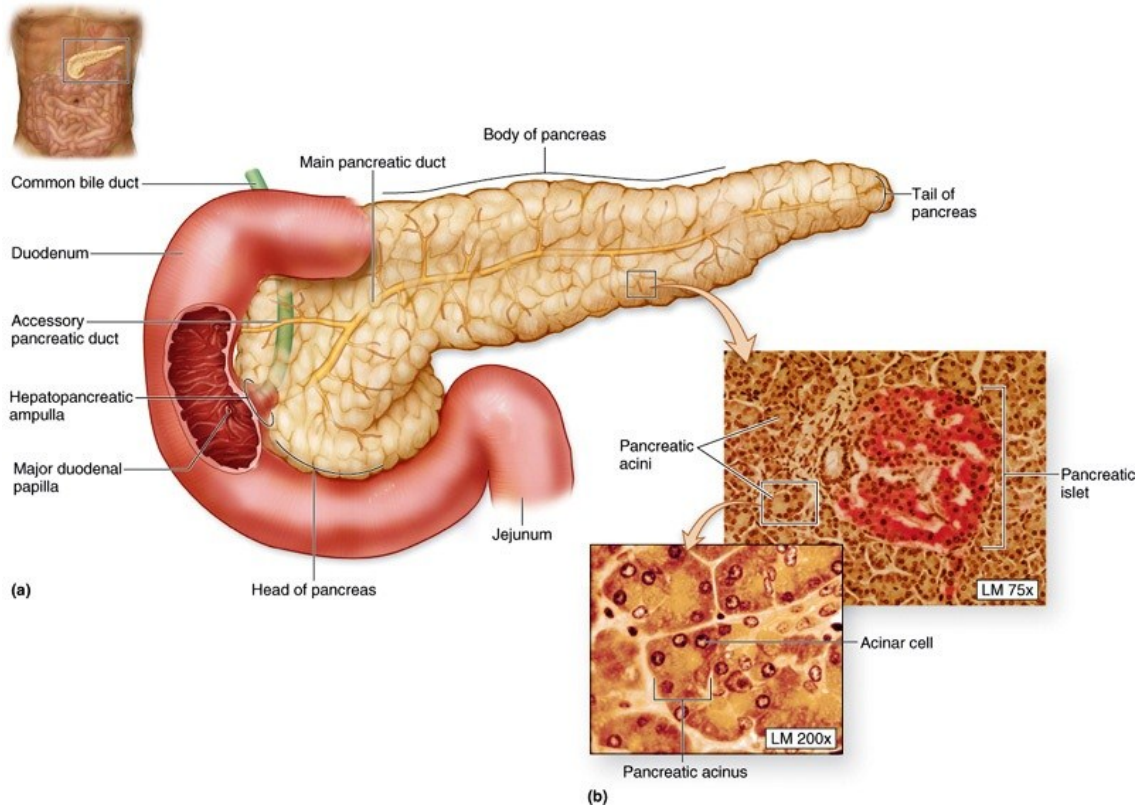


Figure 1 Legend

a) Illustration of the macroscopic anatomy of the pancreas and associated structures

b) Photomicrographs of human pancreatic tissue

[Adapted from http://academic.kellogg.edu/herbrandsonc/bio201_mckinley/Digestive%20System.htm]

Microscopic Structure and Function

The pancreas is a composite gland with both exocrine and endocrine components. It is highly lobulated and invested in a thin loose collagenous capsule which extends as delicate septa between the lobules, providing support for the parenchymal elements. The exocrine pancreas, which forms the bulk of the gland (~80%), is comprised of closely packed secretory acini which drain into a highly branched duct system (See Figure 1).[9] Digestive enzymes are secreted by the pancreatic acini, and large volumes of sodium bicarbonate are produced by centroacinar and ductal cells. The combined product then flows through the pancreatic duct system before emptying into the duodenum. Pancreatic juice is secreted in response to chyme entering the upper portions of the small intestine and is controlled by the vagus nerve and two hormones:

secretin, which stimulates bicarbonate production; and cholecystokinin, which stimulates enzyme secretion.[10]

The endocrine pancreas is composed of isolated clumps of cells, the islets of Langerhan's, which are scattered throughout the exocrine tissue (See Figure 1). The islets are organised around small capillaries which form an insulo-acinar portal system and are comprised of three major types of cells: beta cells that secrete insulin, alpha cells that secrete glucagon and delta cells that secrete gastrin and somatostatin.[9, 11]

CHRONIC PANCREATITIS

Chronic Pancreatitis (CP) is an inflammatory condition that leads to progressive and irreversible destruction of pancreatic tissue. Traditionally CP has been classified as fundamentally different from acute pancreatitis, in which there is a full clinical recovery and pancreatic anatomy and physiology return to normal.[12] However acute, recurrent acute, and chronic pancreatitis are now regarded as a disease continuum.[13] Morphological and functional changes occur during each progressive stage of the disease which pathologically results in fibrosis, with loss of exocrine and endocrine function, causing malnutrition, diabetes and significant pain. In the western, industrialised world chronic excess alcohol consumption is the most prevalent aetiology of CP, however tropical and idiopathic forms are predominant in the developing world.

In recent years cigarette smoking has been recognised as a significant independent risk factor for the development and progression of chronic pancreatitis.[14] This exemplifies the improvements in our understanding of this complex disease that have taken place latterly. However a considerable number of patients still have no identifiable cause for their disease and the exact pathogenesis remains obscure. Perhaps most confusing is the observation that most heavy drinkers do not develop pancreatitis. It seems likely therefore that there is interplay between environmental and genetic factors which determines personal susceptibility to CP. The immune system and its regulation of inflammation therefore represents an interesting area in which to focus research.

Epidemiology

Unfortunately due to the occult nature of the disease, particularly in its early phase, the diagnosis of CP is often difficult. Over previous decades a number of classification systems have therefore been proposed to provide a basis for diagnosis, research and

treatment. The Marseille and Marseille-Rome classifications were based on clinical and pathological criteria, whereas the Cambridge classification relied heavily on morphological features. More recently, detailed clinicopathological systems have incorporated new genetic, aetiological and clinical insights.[15-20] This evolving classification process however has made comparative epidemiology difficult as studies have used numerous different diagnostic criteria, with more recent classification systems being more rigorous and newer diagnostic techniques more sensitive.

The number of large epidemiological studies examining the incidence and prevalence of chronic pancreatitis is surprisingly small. In Europe the overall prevalence is approximately 6 to 7 per 100,000 population.[21, 22] In England and Wales, national statistics for admissions with CP demonstrate a rising incidence over the last half century, with most recent estimates reporting an incidence of 8.6 per 100,000 population.[23, 24] Overall the data suggest that the incidence of chronic pancreatitis is increasing in every nation.[25] It is likely that a rise in their alcohol consumption coupled with improving diagnostic techniques are responsible for the changes seen.

Patients with CP most frequently present in their fifth decade of life. There is a marked gender difference in chronic pancreatitis, which is predominantly a disease of men, with the majority of studies showing that between 73% and 91% of patients are male.[25] This is largely explained by the high proportion of men amongst heavy drinkers. This sex difference is true in all countries. A recent study in Holland has also predicted that there will be an increasing rate of chronic pancreatitis in women due to rising alcohol consumption.[26]

In 1997 in England and Wales, 97 deaths were attributed to chronic pancreatitis. It is therefore clear that chronic pancreatitis is infrequently recorded as the cause of death.[27] Despite this the survival rate for patients with chronic pancreatitis over time is poor. It is high initially, and then decreases significantly: 5 year survival is 97%; 10 year survival varies from 70% to 86.3%; and 20 year survival is between 45% and 63%.[25]

Aetiology and Pathogenesis

In the industrialised western world in the past, the aetiology of chronic pancreatitis was divided into three categories: alcoholic, idiopathic and “other”. This latter category comprised rarer conditions such as hereditary pancreatitis, hypertriglyceridaemia, hyperparathyroidism, autoimmune pancreatitis and various causes of pancreatic duct obstruction, including pancreas divisum. However, advances in the knowledge of the

aetiopathogenesis of chronic pancreatitis led to the development of the TIGAR-O classification system which was proposed in 2001 (See Figure 2).[15]

Figure 2: TIGAR-O Classification System

Toxic-metabolic

- Alcohol
- Tobacco smoking
- Hypercalcaemia (hyperparathyroidism)
- Hyperlipidemia
- Chronic renal failure
- Medication (phenacetin abuse, possibly from chronic renal insufficiency)
- Toxins (organotin compounds (tin hydrocarbons) e.g. DBTC)

Idiopathic

- Early onset
- Late onset
- Tropical (tropical calcific pancreatitis, fibrocalculous pancreatic diabetes)
- Other

Genetic

- Autosomal dominant (cationic trypsinogen, codon 29 and 122 mutations)
- Autosomal recessive/modifier genes (CFTR; SPINK1; cationic trypsinogen (codons 16, 22, 23) and possible α1-antitrypsinogen mutations)

Autoimmune

- Isolated autoimmune chronic pancreatitis
- Syndromic autoimmune chronic pancreatitis (Sjögren's syndrome-, inflammatory bowel disease- and primary biliary cirrhosis-associated chronic pancreatitis)

Recurrent and severe acute pancreatitis

- Post-necrotic (severe acute pancreatitis)
- Recurrent acute pancreatitis
- Vascular diseases/ischaemic
- Post-irradiation

Obstructive

- Pancreas divisum
- Sphincter of Oddi disorders (controversial)
- Duct obstruction (e.g. tumour)
- Pre-ampullary duodenal wall cysts
- Post-traumatic pancreatic duct scars

Figure 2 Legend

TIGAR-O classification system of risk factors and aetiologies of chronic pancreatitis. [Adapted from Etemad et al, Gastroenterology 2001].[15] (DBTC - Dibutyltin dichloride; CFTR - Cystic fibrosis transmembrane conductance regulator; SPINK1 - Serine protease inhibitor kazal type I)

The more recent M-ANNHEIM classification system allows patients to be categorised according to aetiology, clinical stage and severity of disease. This system is based on the assumption that chronic pancreatitis results from the interaction of multiple risk factors (M) which are further sub-categorised into: alcohol consumption (A), nicotine consumption (N), hereditary factors (H), efferent pancreatic duct factors (E), immunological factors (I), and miscellaneous and metabolic factors (M).[28]

Chronic excess alcohol consumption is the most documented and prevalent aetiology of chronic pancreatitis. A clear association between the increasing risk of developing CP compared with the quantity of alcohol drunk was established in 1978 by Durbec and Sarles.[29] As excessive alcohol consumption is the dominant environmental risk factor for CP the proportion of patients with alcoholic chronic pancreatitis is accordingly high in the western industrialised world, comprising 42% – 84.8% of patients in Northern Europe.[25] The quantity and duration of excess alcohol consumption that predisposes to the development of chronic pancreatitis is unknown, however studies have revealed that consumption of greater than 100g alcohol per day leads to an eleven-fold increased risk of developing CP.[30] Furthermore 90% of patients have drunk hazardously for more than ten years with the highest risk found in those that began drinking at a younger age.[22, 31]

Although the clinical, morphological and aetiological characteristics of CP are now well known, the pathogenesis of the disease has remained elusive. Four main hypotheses have emerged over recent decades to explain it: the oxidative stress, toxic-metabolic, stone and duct obstruction, and necrosis-fibrosis theories. The accumulation of new knowledge regarding the cellular, genetic and molecular mechanisms of pancreatic fibrosis, has allowed new pathogenic models to be generated. The most recent model, the sentinel acute pancreatitis event (SAPE) hypothesis,[32] incorporates recent advances with previous theories (See Figure 3). It proposes that in at-risk individuals the pancreatic acinar cells are stimulated by alcohol, oxidative stress and other insults. Fibrosis does not occur at this stage because a pro-fibrotic cellular infiltrate is not yet present. When trypsin activation becomes unregulated, the first episode of acute pancreatitis occurs (the sentinel event). This produces an early and late inflammatory response. The early phase consists of pro-inflammatory cells (neutrophils, lymphocytes and macrophages) and cytokines (TGF-β, TNF-α, IL-6 and others) which attract a distinct, later, anti-inflammatory cellular infiltrate. Pro-fibrotic cells, including pancreatic stellate cells (PSC) constitute the late phase of acute pancreatitis, whose activation sets the stage for the development of pancreatic fibrosis. If inciting factors such as

alcohol and oxidative stress are removed, the pancreas heals to its baseline, normal state. If the acinar cells continue to secrete cytokines in response to oxidative stress, recalcitrant alcohol use, or recurrent bouts of acute pancreatitis, activated PSC continue to respond to cellular signals and deposit collagen, leading to fibrosis and eventually chronic pancreatitis.[32, 33]

Figure 3: Pathogenic Pathways of Chronic Pancreatitis Enumerated by the TIGAR-O Classification

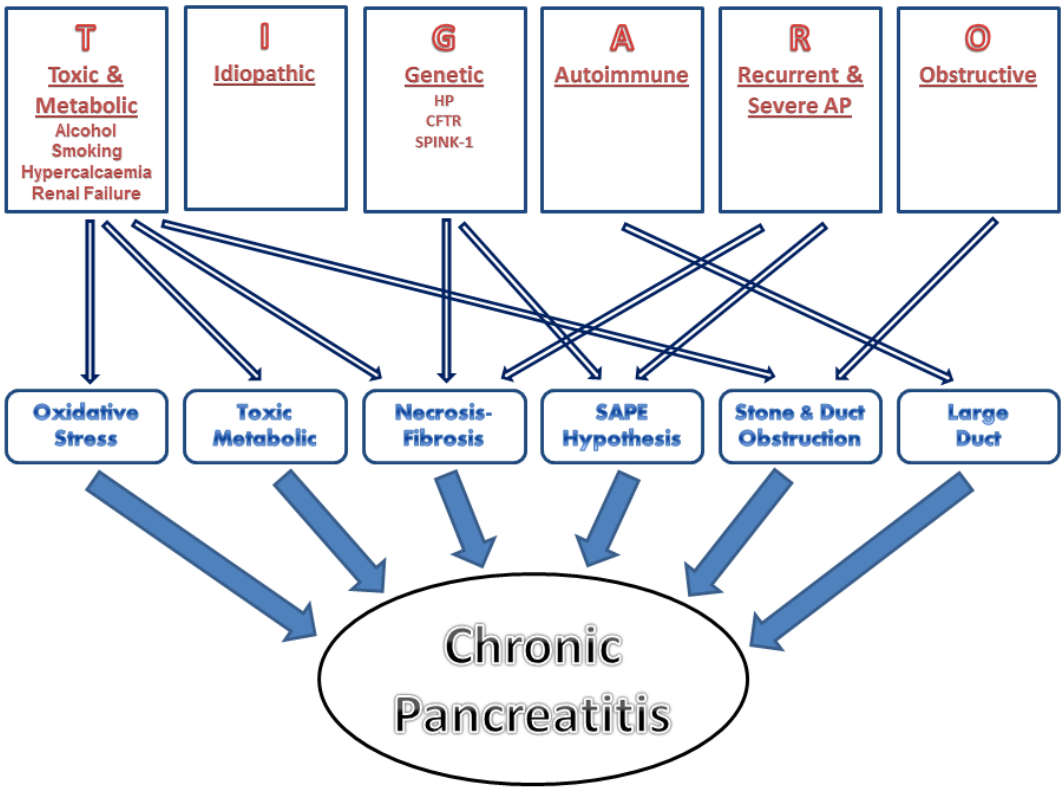


Figure 3 Legend

Pathogenic pathways proposed to each aetiology of chronic pancreatitis enumerated by the TIGAR-O classification.[15] It is likely that different pathophysiological mechanisms may explain the diverse aetiologies of CP. (HP – Hereditary pancreatitis; CFTR - Cystic fibrosis transmembrane conductance regulator; SPINK1 - Serine protease inhibitor kazal type I; AP – Acute pancreatitis; SAPE – Sentinel acute pancreatitis event) [From Stevens et al, Am J Gastroenterol 2004].[33]

Histopathological Changes of Chronic Pancreatitis, Pancreatic Fibrosis and the Role of Pancreatic Stellate Cells

The histopathology changes of chronic pancreatitis are dominated by cellular degeneration, atrophy and fibrosis. There is substantial destruction of the normal ductal epithelia and a prominent chronic inflammatory cell infiltrate is present. Pancreatic fibrosis is the result of increased deposition and reduced degradation of extra-cellular matrix.[34] Pancreatic stellate cells play a central role in the fibrogenic process. In health they display a quiescent phenotype characterised by the presence of vitamin-A containing lipid droplets and function to regulate extra-cellular matrix turnover via their ability to synthesise matrix proteins and matrix degrading enzymes (matrix metalloproteinases, MMP). After injury they activate to a myofibroblastic phenotype (activated PSC, aPSC), proliferating, expressing α-smooth muscle actin (α-SMA) and secreting matrix proteins especially interstitial collagens, which contribute to the fibrotic process.[35, 36] After acute injury, aPSC are lost from the tissue and fibrosis resolves.[37] In CP however, cytokine and growth factor release by damaged resident cells, infiltrating inflammatory cells and aPSC themselves may perpetuate PSC activation leading to dense fibrosis and compromised pancreatic function.[38]

Overview of Immunology in Chronic Pancreatitis

It has been long been considered that pancreatitis is a chemically induced disease, initiated by acinar or ductal damage. This is supported by the fact that a number of disease states exist that lead to CP yet the histopathological process is similar. Studies of tissue from patients with CP reveal an infiltrate of inflammatory cells however they are not well characterised and their precise roles have not been determined. The normal pancreas contains few or no lymphocytes, however during the inflammatory process associated with the development of CP, lymphocytes are recruited into the pancreas in large numbers, along with macrophages, whatever the aetiology.[39] The role of lymphocytes is unclear although evidence is mounting that supports their role in orchestrating an inflammatory response which results in fibrosis. It is also unclear what antigen(s) the lymphocytes are targeting. The following sections will describe an overview of the immune system and discuss the evidence for lymphocytes playing a central role in the development and progression of CP.

THE IMMUNE SYSTEM

The immune system evolved to protect multicellular organisms from pathogens. We are continually exposed to organisms that are inhaled, swallowed, or inhabit our skin and mucous membranes and whether these organisms cause disease is a result of both the pathogenicity of the organism and host defence mechanisms. The immune system is an interactive network of lymphoid organs, cells, humoral factors and cytokines whose essential function of host defence is best illustrated when it goes wrong; under-activity results in the severe infections and tumours of immunodeficiency, over-activity in allergic and autoimmune disease.

Immunity is divided into two parts determined by the speed and specificity of the reaction. These are named the innate and the adaptive immune systems. The innate immune system serves as a first line of defence and involves cellular and molecular mechanisms which become activated upon contact with pathogens and/or cellular damage. An immediate inflammatory reaction is generated as a result of the action of several elements of the immune system including neutrophils, monocytes, macrophages, complement, cytokines and acute phase proteins. The recognition elements of the innate immune system can distinguish well between “self” and pathogens, but are not specialised to distinguish small differences in foreign molecules. In contrast the adaptive immune system responds in a way that facilitates recognition, elimination and memory of the specific invading pathogen. It therefore provides a comprehensive second line of defence to pathogens that evade the innate responses or persist despite them, and in particular it provides for immunological memory. This response consists of antigen-specific reactions through T- and B- lymphocytes. [40, 41]

Leukocytes

All blood cells arise from self-renewing haematopoietic stem cells that allow for the development of erythrocytes, platelets and leukocytes. Several different types of leukocytes exist which can be grouped by the presence or absence of granules in their cytoplasm: granulocytes (polymorphonuclear leukocytes) comprise neutrophils, basophils and eosinophils; whereas agranulocytes (mononuclear leukocytes) include lymphocytes, monocytes and macrophages. Early in haematopoiesis, a multipotent stem cell differentiates along one of two pathways, giving rise to either a myeloid or lymphoid progenitor cell. These progenitor cells are committed to a particular cell lineage whereby lymphoid progenitor cells give rise to B- and T-lymphocytes and natural killer (NK) cells, and myeloid progenitors generate various granulocytes and

monocytes, as well as dendritic cells, erythrocytes and platelets. The lymphoid and leukocytic myeloid cells serve as the effector cells of the immune system, producing a co-ordinated immune response involving both innate and adaptive elements.

Myeloid cells

Several cell types constitute the myeloid cell lineage including mast cells, granulocytes and monocyte macrophages. Mast cells serve as sentinels in tissue, releasing histamine and other inflammatory mediators in response to tissue injury, immunoglobulin (Ig) E receptor cross-linking or activation by complement. Granulocytes on the other hand are found in the blood and are characterised by multi-lobulated nuclei and a granular cytoplasm which contains pre-formed toxic and cytolytic compounds. They are predominantly comprised of neutrophils that are key effectors of innate immunity and function as professional phagocytes, engulfing and destroying damaged cells, cellular debris and pathogens coated with antibodies and complement; but also include eosinophils that primarily function to protect from parasitic infection and basophils that are morphologically and functionally similar to mast cells.

Monocytes leave the bloodstream and enter damaged tissues in response to a range of chemotactic stimuli including damaged cells, pathogens and cytokines. They then undergo a series of changes to become long-lived, activated macrophages that phagocytose pathogens, injured cells and cellular debris, as well as performing the vital acts of presenting antigen to T-cells and releasing complement, cytokines and chemokines, linking the early innate immune response to the later and more sustained adaptive immune response.[40, 41]

Lymphoid Cells

Lymphoid cells constitute up to 40% of the body's white blood cells. They circulate continuously in the blood and lymph and are capable of migrating into and through tissues and lymphoid organs. They may be divided into three categories: innate lymphocytes such as NK cells that are cytotoxic and have the morphology of lymphocytes but do not bear a specific antigen receptor; adaptive lymphocytes such as major histocompatibility complex (MHC) restricted CD4+ and CD8+ T-lymphocytes; and innate-like adaptive lymphocytes which comprise B-lymphocytes that secrete antibodies, and non-polymorphic MHC restricted invariant natural killer T-cells (iNKT) and mucosal-associated invariant T-cells (MAIT).

T-lymphocytes bearing antigen receptors are central cells in adaptive immunity. They are responsible for immunological diversity, specificity and memory, and interact with other cells of the immune system. This interaction involves antigen presentation/recognition, cytokine secretion and destruction of micro-organisms.[40, 41] This thesis centres on the investigation and characterisation of T-lymphocytes in chronic pancreatitis therefore I shall now focus on them.

T-LYMPHOCYTES

There are three well defined subpopulations of T-cells: T-helper (Th), T-cytotoxic (Tc) and T-regulatory (Treg) cells. Th- and Treg- cells may be identified by the expression of the CD4 membrane glycoprotein on their surface, whereas Tc-cells express CD8 on their surface. The ratio of CD4+ to CD8+ T-cells is approximately 2:1 in normal human peripheral blood, but may be altered in disease states.

T-lymphocytes derive their letter designation as they mature in the thymus. During maturation they express a unique antigen-binding molecule, the T-cell receptor (TCR). T-cell receptors recognise antigen only when it is bound to major histocompatibility complex (MHC) molecules. Two main types of MHC molecules exist: Class I (HLA-A, -B, -C in humans); and Class II (HLA-R, -DP, -DQ in humans). CD8+ Tc-cells recognise antigen bound to MHC class I molecules which are expressed on nearly all nucleated cells. The antigens presented are derived from endogenous proteins synthesised by the cell (e.g. self-proteins and virus-derived antigens). Hence CD8+ Tc-cells preferentially recognise virus infected or altered self cells that present an altered pattern of self peptides on their MHC class I molecules. When CD8+ Tc-cells are exposed to infected or dysfunctional cells, they release cytotoxins such as granzymes and perforin which form pores in the target cell's membrane and activate the caspase cascade, eventually resulting in cell death by apoptosis. They can also induce apoptosis by the interaction of target cell Fas receptors (CD95) with Fas ligand.[40] Tc-cells display a number of different cell surface markers and have different functional capacities. A significant proportion are intra-epithelial lymphocytes that express CD161+ and exist in the intestinal epithelium and liver.[42] They are largely CD45RO+ central memory T-cells and effector cells that secrete high levels of IFN-γ, TNF-α and IL-17.[42, 43]

CD4+ T-cells recognise antigen presented by MHC class II molecules. These are only expressed on a restricted number of cell types: professional antigen-presenting cells including dendritic cells, macrophages and B-cells. The antigens presented are derived from exogenous proteins taken up by endocytosis, which are then digested in lysosomes before loading onto the MHC class II molecule. This process allows CD4+ T-cells to activate and direct appropriate immune cellular responses to pathogens. They are essential in determining B-cell antibody class switching, in the activation and growth of Tc-cells, in maximising the bactericidal activity of phagocytes such as macrophages, and to orchestrate the full panopoly of immune responses through their production of cytokines and chemokines.[40]

T-Helper and T-Regulatory Cells

CD4+ T-cells represent a series of distinct cell populations with different functions. Some of these CD4+ T-cell populations are discrete lineages of cells already distinguished from one another when they leave the thymus, such as natural T-regulatory (nTreg) cells, whilst others represent alternative pathways of differentiation of naïve T-cells.[44] Naïve conventional T-cells have four (and maybe more) possible fates determined by the pattern of signals they receive during their initial interaction with antigen. These populations are Th1, Th2, Th17, and induced T-regulatory (iTreg) cells (See Figure 4).

Figure 4: Summary of the Possible Fates of Naïve CD4+ T-Helper Cells

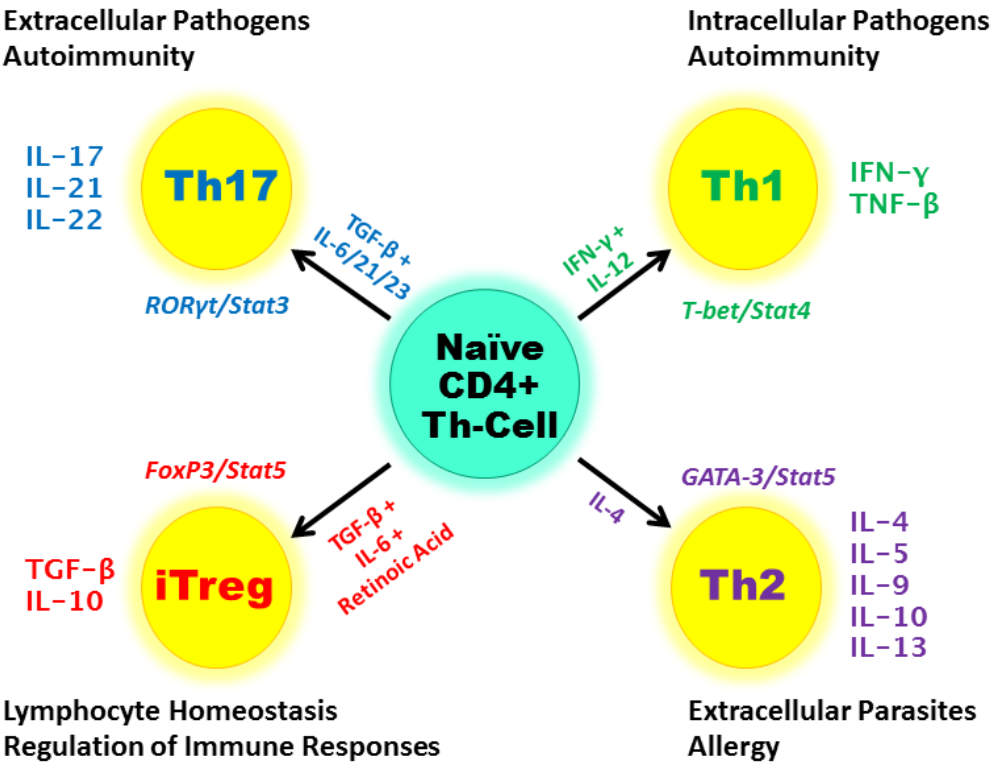


Figure 4 Legend

Summary of the four T-helper cell fates: The function (black lettering), unique products (adjacent to cell), characteristic transcription factors (above/below cell), and cytokines critical for the determination of their fate (parallel to black arrows) are shown. [Adapted from Zhu et al, Blood 2008] [45]

Th1 and Th2 Cells

In 1989 Mosmann and Coffman introduced the concept of distinct populations of T-helper cells, based on the types of cytokines they produced when stimulated to differentiate. They named the most identifiable populations Th1 and Th2 cells, which had reciprocal patterns of immunity: Th1 cells were regarded as critical for cell mediated immunity directed against intracellular micro-organisms; and Th2 cells were considered essential for humoral immunity aimed at protection from extracellular pathogens, including parasites.[46] Th1 cells mainly produce interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), which are important for macrophage activation. Th2 cells produce interleukin (IL)-4, and additionally produce IL-5, Il-9, IL-10 and IL-13, which induce IgE production and recruit eosinophils to sites of inflammation (See Figure 4).[47, 48]

It was soon discovered that the classification system of T-helper cell subsets in terms of promoting either delayed-type hypersensitivity reactions or humoral immunity was over-simplified as IFN-γ could induce class switching of certain B-cell antibodies, and thus was essential for some humoral responses.[49] The function of Th1 cells therefore seemed related to stimulating phagocyte-mediated defence against infections by activating macrophages and promoting opsonisation. However, although the pro-inflammatory properties of Th1 cells and their secreted cytokines IFN-γ and TNF-β are well suited for pathogen clearance by recruiting and activating inflammatory leukocytes, they can also cause tissue damage and elicit unwanted inflammatory disease and self-reactivity. It has been shown that Th1 cells and IFN-γ contribute to inflammatory diseases such as inflammatory bowel disease and graft-versus-host disease, as well as autoimmune disorders such as type-1 diabetes mellitus and rheumatoid arthritis.[50-55]

The Th2 response is usually associated with humoral responses during which pathogen-specific immunoglobulins are generated to neutralise foreign organisms. In particular Th2 cell production of IL-4 is the major inducer of B-cell class switching to IgE production, and is therefore key to mast-cell mediated reactions; and IL-5 is the principal eosinophil-activating cytokine.[56, 57] Thus Th2 cells are important to resist extracellular pathogens such as helminths and nematodes.[58] Th2 cells are also important for mucosal immunity in the lung and chronic inflammatory airway diseases such as atopic asthma are associated with an over-exuberant Th2 response.[59, 60] It should be noted that several cytokines produced by Th2 cells also have anti-inflammatory actions: IL-4 and IL-13 antagonise the activation of macrophages by IFN-γ; and IL-10 suppresses numerous macrophage responses. Th2 cells may thus inhibit

acute and chronic inflammation, including delayed-type hypersensitivity responses. It is therefore possible that the physiological function of Th2 cells is not just as an effector but as a regulator of the immune response, whereby the late appearance of Th2 cells may limit the injurious consequences of Th1 cell mediated immunity.[61]

Th17 Cells

The advent of sensitive intracellular cytokine assays broadened our understanding of T-helper cell subsets as many T-cells were identified that did not fit into the proposed schemata according to their cytokine secretion profile. T-cells that preferentially secrete IL-17 rather than IFN-γ or IL-4 were named Th17 cells. They appear to play a critical function in protection against extracellular bacteria and fungi, and can rapidly initiate an inflammatory response that is dominated by neutrophils.[62-64] Immunity mediated by Th-17 cells is particularly important at epithelial and mucosal surfaces, as indicated by their pattern of chemokine receptors (CCR4 and CCR6) and effector cytokines secreted (IL-17, IL-21 and IL-22).[62] Upregulated Th17 responses or overwhelming IL-17 production from T-cells is associated with chronic inflammation. Most parenchymal cells express IL-17 receptors, and signalling through these receptors induces the production of pro-inflammatory factors such as IFN-γ, IL-1, IL-6, tumour necrosis factor, and matrix metalloproteinases. These then in turn activate a positive feedback loop committing naïve T-cells to a Th17 lineage, in addition to promoting effector T-lymphocyte chemo-attraction, tissue destruction and degradation of extracellular matrix.[63-65] Th17 cells are responsible for, or participate in, the induction of many autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis, as well as inflammatory bowel disease, whose development was originally attributed to Th1 cells before the discovery of Th17 cells.[66-69] CD161 expressing Th17 cells that are activated by IL-23 and are imprinted for gut homing, as indicated by high levels of CCR6 and Integrin-β7 expression, have also been shown to mediate destructive tissue inflammation in the inflammatory bowel disease Crohn's disease.[70]

Treg Cells

CD4+ Treg cells are a unique subset of T-lymphocytes that play an important function in maintaining immune homeostasis and protecting the host against autoimmune disease by suppressing harmful immunopathological responses to self or foreign antigen.[44, 71] The transcription factor Forkhead box P3 (FoxP3) is essential in establishing a functional Treg cell lineage although it may be transiently expressed on activating T-cells *in-vitro*.[72-74] In addition, the majority of Treg cells constitutively express: high levels of IL-2 receptor α-chain (CD25) molecules, which are also present

on activated T-cells; cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), an immunoglobulin associated with transmission of inhibitory signals to T-cells; and glucocorticoid-induced TNF-receptor family-related gene (GITR), which has been shown to be involved in inhibiting the suppressive activity of T-regulatory cells and extending the survival of T-effector cells.[75-78]

nTreg cells are thymic derived FoxP3+ cells that appear to allow a balanced response to environmental antigen and have been shown to prevent inflammatory bowel disease in rodent models.[79] Functionally, they produce IL-10 and membrane-bound forms of TGF-β, and inhibit the proliferation of CD4+CD25- and CD8+ lymphocytes in a contact dependent manner.[80-82] Despite the fact that nTreg cells are central to the maintenance of self-tolerance, this negative regulatory activity may have adverse consequences, in particular in suppressing appropriate responses to tumours. Tumours are assisted in evasion of immune destruction by actively inducing immune tolerance through the recruitment of $CD4^+CD25^+FoxP3^+$ nTreg cells. High nTreg cell numbers have been found in many tumours, including breast and pancreatic carcinoma.[83-85] In addition increased numbers of nTreg cells are present in the peripheral blood of patients with advanced pancreatic carcinoma.[84, 86]

iTreg cells have become established as an inducible cell population that phenotypically resemble nTreg cells, although the full extent of differences and similarities between iTreg and nTreg cells has yet to be defined.[87] It is possible that iTreg cell development is driven by the need to maintain a non-inflammatory environment in the gut by suppressing immune responses to environmental and food allergens, and to decrease chronic inflammation, whereas nTreg cells prevent autoimmunity and raise the activation threshold for all immune responses. So far no reliable marker has been shown to distinguish iTreg cells from nTreg cells. Functionally iTreg cells also produce large amounts of IL-10 and TGF-β but are differentiated from naïve T-cells in the presence of TGF-β following TCR stimulation in the periphery.[88] Given that there is such similarity between nTreg and iTreg cells they will be considered as a single population in this thesis unless otherwise stated.

Generation of T-Helper Cell Subsets

T-helper cells are derived from pre-cursors whose differentiation is influenced by the manner and environment in which they are stimulated.[89] The cytokine milieu during priming with antigen and the consequent activation of specific transcription factors are two key elements controlling T-helper cell differentiation of naïve CD4+ T-cells. A

distinct set of cytokines promotes the differentiation process for each lineage: IL-12 and IFN-γ for Th1; IL-4 for Th2; TGFβ / (IL-6, IL-21, IL-23) for Th17; and TGF-β / IL-6 for iTreg cells. The transcription factors that govern the differentiation of these cells are also well defined: T-bet / Stat4 for Th1; GATA-3 / Stat5 for Th2; RORγt / Stat3 for Th17; and FoxP3 / Stat5 for iTreg cells (See Figure 4).[90]

Each T-helper cell cytokine also serves as its own paracrine growth factor and promotes differentiation of naïve T-cells to that subset.[91] Furthermore the cytokines released by each subset may regulate another's development, such that Th1 produced IFN-γ promotes Th1 and inhibits Th2 cell development, whereas IL-10 produced by Th2 cells inhibits Th1 activation.[92] These regulatory processes promote development of specific T-helper cell immune responses that may therefore become progressively polarised.

T-Helper Cell Plasticity

In immune responses to infection and in autoimmune disease models, Th1 and Th17 cells often develop simultaneously. In mice lacking T-bet, a transcription factor required for Th1 cell differentiation and IFN-γ production, increased levels of IL-17 secreting cells were observed compared to wild-type mice and more severe autoimmune disease.[63, 93] Furthermore T-bet appears to suppress RORγt expression, a transcription factor central to Th17 cell development. These results suggest that T-bet might serve as a negative regulator for Th17 cell differentiation, implying that master regulator genes shape immune responses by simultaneously activating one genetic program while silencing the activity of competing regulators in a common progenitor cell.[94]

However *in vitro* generated Th17 cells are incapable of maintaining their cytokine expressing capabilities and can be converted into Th1 cells in appropriate environments.[95-97] This suggests that Th17 cells are not terminally differentiated and require specific signals to sustain their program. Although re-differentiation of Th17 cells has been observed in several circumstances the physiological relevance, significance and regulatory mechanisms governing differentiation are still unclear.

Reciprocal iTreg and Th17 Differentiation

Despite the fact that naïve T-cells can be converted to iTreg or Th17 cells by TGF-β, further exogenous factors also dictate their fate. Retinoic acid has been shown to be a key regulator of TGF-β dependent iTreg cell induction whereby it is capable of inhibiting

IL-6 driven development of a Th17 phenotype and promoting iTreg cell differentiation.[98] Furthermore there is evidence that iTreg cells are increased within pancreatic tumours by a mechanism which seems dependent on TGF-β receptor expression and the presence of tumour derived TGF-β.[99, 100] It is interesting that vitamin A (retinoic acid is the oxidised form of vitamin A) is lost from PSC following activation and that they also produce TGF-β.[38] These events may therefore have a function in controlling the immune response to inflammatory stimuli.

Tissue Repair, Fibrosis and the Role of T-Helper Lymphocytes

Tissue repair involves two distinct stages: fibrosis, in which connective tissue replaces normal parenchymal tissue; and a regenerative phase, in which injured cells are replaced by cells of the same type and there is no lasting evidence of damage. In most cases, both stages are required to slow or reverse the damage caused by an injurious agent. Improperly regulated tissue repair causes a progressive, complex, multi-stage scarring process that is frequently associated with repeated injury and chronic inflammation, and results in replacement of parenchymal cells with extra-cellular matrix (ECM), impairing organ function.[40]

Fibrosis is regulated and governed by the interaction between fibroblasts, macrophages and other immune cells. Adaptive immune responses can amplify, sustain or suppress the fibrotic process, particularly in chronic progressive disease. The role of T-helper cell cytokines has particular significance in this respect. As previously discussed the Th1/Th2/Th17/Treg paradigm can be focused on different features: the ability of their respective cytokines to drive cell-mediated or humoral responses; the reciprocal models of T-helper cell differentiation; initiation or suppression of harmful immunopathological responses to self or foreign antigen; and counter-regulatory immune responses through pro- and anti-inflammatory actions. These however ignore the underlying role of Th2 cells as important regulators of ECM remodeling. Th2 cells can directly promote fibrosis by stimulating fibroblasts with IL-4 and IL-13.[101] Furthermore TGF-β produced by activated Treg cells also promotes collagen synthesis in fibroblasts, as well as suppresses inflammation.[102]

Whilst Th2 responses promote collagen deposition, Th1 responses inhibit it, indicating opposing actions in tissue repair (See Figure 5). This might explain the frequent bimodal nature of immune responses in general. Initially, the immune response to invading pathogens or chronic irritants is often dominated by Th1 cells. However, if the stimulus persists, immune suppressive mechanisms need to be activated to prevent

excessive damage to host tissues. In these circumstances, Treg cells and Th2 cytokines often collaborate to suppress the Th1 response and more importantly promote the mechanism of wound healing.[103] Hence the overriding survival mechanism when immune responses enter the chronic phase may result in persistent healing and fibrosis. Teleologically this is a viable compromise for the individual because it might be the only way to ensure long-term survival.

Figure 5: The Opposing Roles of Th1 and Th2 Cells in Tissue Fibrosis

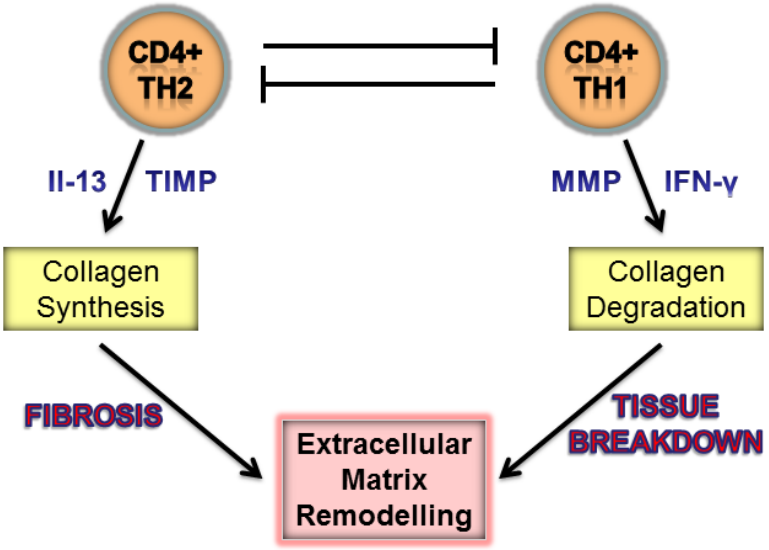


Figure 5 Legend

The Th1 and Th2 cell cytokines regulate collagen synthesis by fibroblasts. This is achieved by controlling the balance of matrix metalloproteinase (MMP) and tissue inhibitor of matrix metalloproteinase (TIMP) production, thereby modulating the rates of collagen degradation and synthesis respectively. [Adapted from Wynn, Nat Rev Immunol 2004][103]

Mucosal-Associated Invariant T-Cells

Conventional T-lymphocytes display a highly diverse repertoire of antigen receptors which enables them to respond to a wide variety of antigens presented by MHC molecules. During development, interactions between T-cells and selecting MHC class I and II molecules present on thymic epithelial cells, leads to the specific features of CD4+ and CD8+ T-cell lineages. However, two “innate-like" T-lymphocytes, the invariant natural killer T-cell (iNKT) and mucosal-associated invariant T (MAIT) cell follow different ontogenic pathways with selection by non-polymorphic MHC class Ib molecules.[104] MAIT cell selection and expansion occurs on thymic B-cells expressing

the MHC class I related molecule MR1 and is dependent on the presence of commensal flora but independent of a transporter associated with antigen processing (TAP). MAIT then migrate from the thymus and become enriched in the intestinal lamina propria and mesenteric lymph nodes. They are also numerous in the peripheral blood of healthy subjects, representing up to 10% of CD4- CD8- double negative (DN) and CD8+ lymphocytes.[104-106]

Human MAIT express a highly conserved TCR-α chain consisting of an invariant TCR Vα7.2-Jα33 re-arrangement paired to a limited set of TCR-β chains.[105-108] MAIT are present in small numbers in cord blood and display a naïve phenotype. They then expand after birth and acquire an effector-memory phenotype ($CD161^{+}CD45RA^{-}CD45RO^{+}CD95^{hi}CD62L^{lo}$) and have immediate effector functions after stimulation, such as cytokine secretion. In particular they have been found to secrete IFN-γ, IL-4, IL-5, IL-10, IL-17 and Granzyme-B following TCR ligation (See Figure 6). [109, 110]

MAIT seem to be involved in immune regulation as well as mucosal anti-bacterial host defence. They have recently been found to detect a wide variety of bacteria: the gram-negative bacilli *Escherichia coli*, *Pseudomonas aeroginosa* and *Klebsiella pneumonia*; the gram-positive bacilli *Mycobacterium tuberculosis* and *Lactobacillus acidophilus;* and the gram-positive cocci *Staphylococcus aureus* and *Staphylococcus epidermidis*. Interestingly fewer MAIT cells are found in the peripheral blood of patients with *Mycobacterium tuberculosis* infection compared to healthy controls, and instead MAIT are enriched in infected lung lesions. Furthermore, MAIT cell activation requires cognate interaction between its invariant TCR and MR1, which presents the bacteria-derived ligand. Viruses and lipopolysaccharide (LPS) did not activate MAIT cells. These results indicate that MAIT cells are recruited and activated at the site of infection and have specific antibacterial properties.[111, 112]

There is only a minimal literature on the potential role of MAIT cells in non-infectious disease, however recent work has assessed their role in multiple sclerosis (MS). The frequency of MAIT cells in peripheral blood was significantly reduced in MS patients in remission and even more profoundly reduced in those with relapse. Furthermore MAIT cells isolated from patients with MS showed a suppressive activity against IFN-γ production by T-cells *in vitro*. This suppression required cell contact but was independent of IL-10 and the presence of B-cells. These results suggest an immune-regulatory role of MAIT cells in MS through suppression of pathogenic Th1 cells.[113]

Figure 6: The Main Features of Mucosal Associated Invariant T-Cells

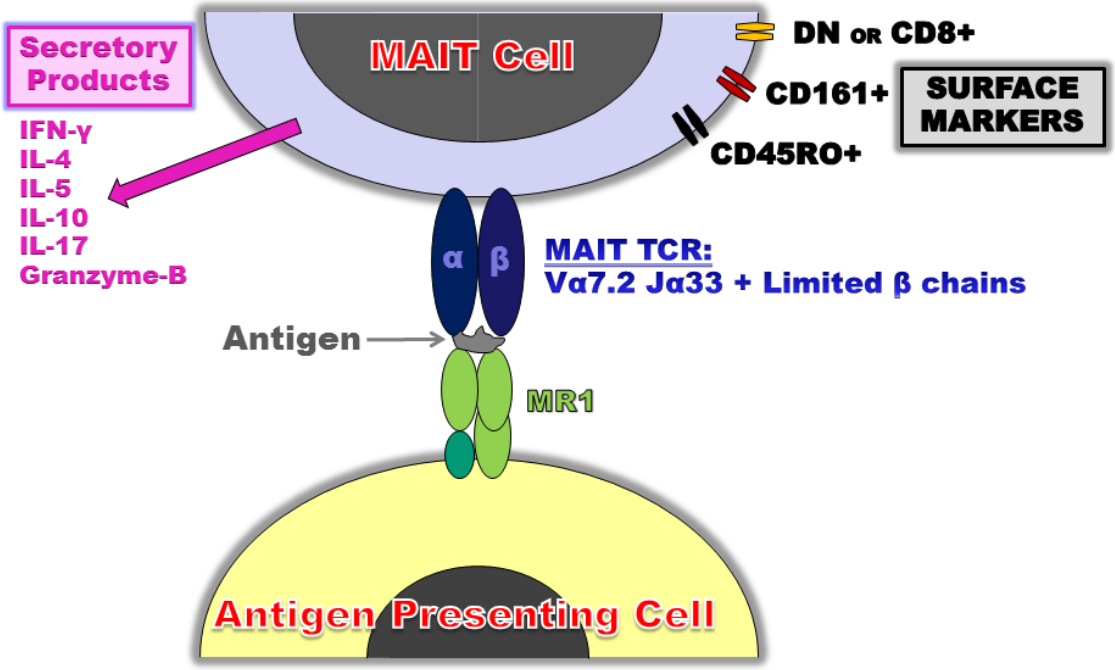


Figure 6 Legend

Diagrammatic representation of the cell surface markers, secretory products and invariant T-Cell Receptor (TCR) of Mucosal-Associated Invariant T (MAIT) Cells and their interaction with MR1 expressing antigen presenting cells. The nature of the MR1 presented antigen is still unknown.

THE PATHOGENESIS OF CHRONIC PANCREATITIS: EVIDENCE FOR A ROLE OF T-LYMPHOCYTES

The normal pancreas contains few or no lymphocytes, however during the inflammatory process associated with the development of CP, lymphocytes are recruited into the pancreas in large numbers along with macrophages whatever the aetiology.[39] The role of lymphocytes is unclear although evidence is mounting that supports their role in orchestrating an inflammatory response which results in fibrosis. It is also unclear which antigens the infiltrating lymphocytes recognise and which homing markers they express that allow recruitment to inflamed pancreatic tissue.

Lymphocyte Homing to the Pancreas

Lymphocyte recirculation and specific antigen-driven recruitment and homing to sites of inflammation are critical to the surveillance and effector functions of the immune system. Naive T-lymphocytes are primed in secondary lymphoid organs such as lymph nodes and return to the circulation via the right lymphatic and thoracic ducts. They then migrate through post-capillary venules to infiltrate target tissues and reach antigenic sites. Antigen is presented to T-lymphocytes by dendritic cells that reside within lymph nodes. The local micro-environment within the lymph nodes and the presentation of antigen allows imprinting of the homing properties to the T-cells. Pancreatic lymph nodes are in an unusual situation as they sample self-antigens from the pancreas and foreign antigens from the gastrointestinal tract and peritoneum. In essence therefore pancreatic lymph nodes can be considered a gut-associated lymphoid tissue (GALT) in their ability to present gastrointestinal antigens. Non-specific perturbations in gastrointestinal physiology may therefore promote a response from T-lymphocytes that is detrimental to the pancreas.[114]

Although there are a multitude of molecules involved in lymphocyte interactions with vascular endothelial cells at different steps of the adhesion cascade, only a few of them are responsible for the mucosal specificity of the lymphocyte homing process. Integrins are heterodimeric cell surface expressed adhesion molecules composed of alpha and beta subunits. During T-cell activation the expression of integrins changes to promote entry of T-cells into non-lymphoid sites. Mucosal addressin cell adhesion molecule - 1 (MAdCAM-1), the integrin α4β7 ligand, is specifically expressed on the vessels of mesenteric lymph nodes and Peyer's patches. Expression of integrin α4β7 on T-cells is thus associated with preferential trafficking to the intestine.[115-117] The pattern of adhesion molecules and chemokine receptors expressed on T-lymphocytes which

govern homing to the pancreas in chronic pancreatitis is currently not known. This may represent a unique pattern or be similar to that for the gut, as demonstrated by animal models of diabetes mellitus.

Pancreatitis and Diabetes Mellitus

The high prevalence of diabetes mellitus (DM) has led to a large volume of research such that the immune mechanisms governing the pathogenesis of islet cell destruction are quite well defined. In comparison, immunological aspects of chronic pancreatitis are poorly understood. Pancreatic islets and the exocrine pancreas are derived from a common embryological bud but the islets are probably immunologically distinct. This is supported by the observation that the inflammatory cell infiltrate seen in type-1 DM, which is predominantly composed of T-lymphocytes and macrophages, causes islet cell destruction without causing significant acinar cell damage.[118]

Interestingly however, in both early and late onset diabetes mellitus (type 1 and 2 DM) functional pancreatic insufficiency may be found as demonstrated by detection of a low faecal elastase. Post-mortem studies on these patients also reveal a degree of pancreatic exocrine fibrosis.[119] The converse is also true: patients with extensive and late stage chronic pancreatitis develop diabetes mellitus (classified as type 3 DM) brought about by bystander islet cells destruction following significant fibrosis and exocrine cell loss.[120] These combined findings support the notion that the two functional and histological compartments of the pancreas are immunologically distinct but bystander injury may occur.

Although the effects of pancreatic lymphocyte infiltration differ according to the compartment in which the initiating stimulus originated, the homing markers the lymphocytes express may be the same. In mouse models of diabetes, T-lymphocytes which are imprinted to home to pancreatic islets are primed in pancreatic lymph nodes and have a phenotype similar to that for the gut, expressing integrin α4β7.[121-124] Lymphocytes that home to pancreatic exocrine tissue may therefore express a similar integrin and chemokine receptor pattern though critical differences will also likely exist that explain the immunologically distinct nature of the two compartments.

Animal Models of Chronic Pancreatitis

A number of animal models of chronic pancreatitis have yielded valuable information regarding the potential role of the immune system in the development of chronic pancreatitis. Unfortunately however their scope is limited and their relevance to human

disease is questionable: Immunologically mediated animal models of human disease are often dependent on autoimmune mechanisms or they spontaneously emerge following compromise of the immune system; and chemically induced models have been inconsistent. Regardless of these pitfalls they still serve to provide an opportunity to explore immunological mechanisms of pancreatic injury.

In chemically induced rat models of pancreatitis using intravenously injected dibutylin dichloride (DBTC), acute non-specific inflammation is followed by infiltration and activation of CD4+ and CD8+ T-cells. Additionally memory type lymphocytes persist and there is enhanced expression of IFN-γ and IL-2 in pancreatic tissue.[125] It has also been shown that pancreatic inflammation may be reduced and T-cell infiltration inhibited by administering immunosuppressive drugs to Wistar Bonn/Kobori (WBK) rats that spontaneously develop chronic pancreatitis similar to autoimmune pancreatitis. WBK rats administered Tacrolimus, an immunosuppressive drug that inhibits T-cell IL-2 production (which stimulates the growth, differentiation and survival of antigen-selected cytotoxic T-cells), developed an attenuated form of chronic pancreatitis. In untreated animals CD8+ T-cells were abundant in the fibrotic tissue where there was associated loss of acinar cells. Repeated dosing with Tacrolimus completely prevented acinar cell apoptosis and suppressed CD4+ and CD8+ T-lymphocyte infiltration.[126] Using the same model the authors demonstrated that FTY720, a novel synthetic immunosuppressant that decreases peripheral blood lymphocytes by accelerating their homing to peripheral and mesenteric lymph nodes and Peyer's patches, also attenuated pancreatic inflammation and fibrosis by preventing T-cell infiltration and reducing expression of IFN-γ and TGF-β.[127]

In an alternative mouse model of pancreatitis which was induced using alcohol and/or lipopolysaccharide, the inflammatory cell infiltrate was also found to be predominantly composed of CD4+ and CD8+ T-cells. Interestingly and most importantly severe combined immunodeficiency (SCID) mice treated with alcohol and LPS only developed pancreatitis when they were reconstituted with CD4+ but not CD8+ T-cells.[128] Conversely in studies of MHC class II deficient mice initially bred to investigate intestinal inflammation but in whom a wasting disease was noted and found to be related to pancreatic atrophy, a peri-ductal T-lymphocyte infiltration composed predominantly of CD8+ T-cells was initially observed which was followed by acinar cell destruction. Adoptive transfer of isolated CD8+ T-cells into athymic mice confirmed that this immune-based model of pancreatitis was orchestrated by CD8+ T-cells.[129] These

two mouse models of pancreatitis suggest that both CD4+ and CD8+ T-cells can play important roles in the pathogenesis of chronic pancreatitis.

Analysis of Human Chronic Pancreatitis Tissue

Analysis of tissue from human patients with chronic pancreatitis is limited as only a minority undergo surgery and the tissue removed often represents end-stage disease. We therefore have limited insight into the true mechanisms governing disease progression in CP, although the tissue analysed can provide useful indications of the likely inflammatory process. The majority of previous work has assessed pancreatic tissue as a whole using either immunohistochemistry or molecular biology techniques. Few have isolated lymphocytes from pancreatic tissue and analysed them, providing an opportunity for novel techniques to be established and new research to be undertaken. Our understanding of the role of lymphocytes in CP therefore currently remains in its infancy.

In 1990 Bedossa *et al* published the first work that analysed normal pancreatic and chronic pancreatitis tissue using immunohistochemistry. They found that normal pancreatic tissue contained few lymphocytes, which were almost exclusively T-cells, and were equally distributed between CD4+ and CD8+ subsets. Pancreas infiltrating T-lymphocytes in CP on the other hand were found to be predominantly CD8+ (n=16).[130] These findings were contradicted by Emmrich *et al* in 1998, who also analysed tissue using immunohistochemistry, and found that the proportion of CD4+ to CD8+ T-cells was nearly identical in both normal pancreatic and chronic pancreatitis tissue (n=7).[131] Further immunohistochemical analysis of CP tissue was undertaken by Shrikhande *et al* who looked for differences between alcoholic, idiopathic and tropical chronic pancreatitis using immunohistochemistry (n=43). They found that CD4+ T-cells were slightly more prevalent than CD8+ in CP tissue. No significant quantitative difference in the specific cellular infiltrates of CD4+, CD8+ and CD45+ T-cells was observed between the three different groups, who also displayed similar histological features and a comparable inflammatory cell reaction.[39] These findings indicate that the disease, independent of the underlying aetiology, reaches a common immunological stage beyond which it appears to progress as a single distinctive entity.

Ebert *et al* analysed frozen pancreatic tissue sections using the novel technique of multiepitope immunofluorescence, allowing the identification of multiple cell surface markers on T-cells and thus improving their characterisation. They demonstrated that CP tissue contained numerous T-cells, which were predominantly CD4+, with a CD4 to

CD8 ratio of 2.4:1. Furthermore CD8+CD103+ lymphocytes, analogous to intraepithelial lymphocytes (IEL), were observed peri-ductally.[132] Interestingly the vitamin A metabolite retinoic acid in combination with TGF-β induces the differentiation of naïve CD4+ T-cells into iTreg cells that strongly express CD103 and CCR9, indicating a gut homing phenotype.[98] The fact that activated pancreatic stellate cells lose vitamin A and produce TGF-β upon activation [38] suggests a possible role for these cells in the generation of peri-ductal CD8+CD103+ iTreg cells.

In 1997 Robert Hunger, Markus Buchler and Helmut Friess, were the first to isolate lymphocytes from pancreatic tissue of patients with alcoholic CP and perform analysis using flow cytometry. The majority of lymphocytes isolated were CD4+ T-cells (30.9%, SD +/- 11.6%) and CD8+ T-cells (26.1%, SD +/-12.0%). They also analysed paraffin embedded tissue using in-situ hybridisation and found a significant increase in perforin mRNA and protein expressing cells in the pancreata of patients with CP compared to controls. Perforin is a specific in-vivo activation marker for cytotoxic cells, such as CD8+ T-cells, suggesting that they contribute to tissue destruction in CP.[133]

From these studies we may conclude that the ratio of infiltrating CD4:CD8 T-cells is most likely to be relatively equal and the discrepancies observed are probably related to small sample sizes and employment of different techniques. Furthermore, the detection of perforin in CP tissue supports the concept that cell mediated cytotoxicity is important in the pathogenesis of CP. The development of CP is a multi-stage process and cell mediated immunity likely only represents one stage. It might however represent the common pathway of immune mediated tissue destruction that results in chronic pancreatitis regardless of the aetiology. The further characterisation of CD4+ T-cells present in chronic pancreatitis tissue clearly therefore merits further investigation.

Alterations in the Distribution and Function of Peripheral Blood Lymphocytes

Analysis of peripheral blood mononuclear cells (PBMC) has, as for pancreatic tissue, revealed conflicting results and therefore the true immunological perturbation remains unclear. Initial data demonstrated a reduction in the percentage of CD8+ and CD25+ T-lymphocytes in the peripheral blood of patients with chronic pancreatitis compared to healthy controls (n=11). PBMC from CP patients also produced higher levels of both the pro- and anti-inflammatory cytokines TNF-α and IL-10 when stimulated with IL-2 or PMA and ionomycin.[134]

Gansauge *et al* then reported an increase in the absolute numbers of CD4+ and CD8+ T-cells in the peripheral blood of CP patients compared to healthy controls (n=48). The authors did not comment on whether the proportion of CD4+ and CD8+ T-cells was altered between groups. They did however assess the blastogenic reaction of whole blood to phytohaemagglutinin (PHA) and anti-CD3 and found that the response of peripheral blood lymphocytes was significantly attenuated in CP patients. One year after pancreatic resection the absolute numbers and blastogenic response of T-cells returned to normal compared to pre-operative values.[135] These findings suggest that chronic inflammatory processes are the reason for the reduced proliferative capacity of peripheral blood lymphocytes as surgical removal of the focus of inflammation returned the inflammatory response to normal.

Following this Bhatnagar *et al* demonstrated a reduction of CD4+ and an increase in CD25+ T-cells in the peripheral blood of patients with CP compared to controls (n=27). They also noted reduced HLA-DR expression on monocytes, indicating altered functional capacity to present antigen. Following stimulation of PBMC with lipopolysaccharide (LPS), CD3+ T-cells from CP patients secreted higher concentrations of IL-2 and IFN-γ and lower concentrations of IL-4, indicating polarisation of T-cells towards the Th1 phenotype. However it must be noted that the authors did not specifically identify CD4+ T-cells as the source of IFN-γ, a cytokine that can also be produced by CD8+ T-cells.[136]

A smaller (n=18) but more focused study evaluated the ratio of naïve to memory T-lymphocytes in the blood of CP patients. This demonstrated an increase in both CD4+ and CD8+ CCR7+CD45RA- central memory T-cells in CP compared to healthy controls. The relative increase in central memory T-lymphocytes was found to persist in patients after pancreatic resection suggesting that the increase in central memory T-lymphocytes maintains the inflammatory process in CP.[137] Finally a study comparing patients with pancreatic adenocarcinoma, CP and healthy control subjects found an increase in the total number of CD4+ lymphocytes but no change in CD8+ lymphocytes in CP compared to controls.[138]

In conclusion it is unclear whether the proportion of CD4+ or CD8+ T-cells changes in the peripheral blood of patients with CP. It does however seem that there is an increase in the number of CD25+ activated and CCR7+CD45RA- memory T-cells. The response of PBMC and isolated lymphocytes to stimulation suggests a deviation towards a pro-inflammatory phenotype, which may be Th1 predominant, however the

attenuated blastogenic response found in CP seems an unusual finding as an enhanced cytokine response would seem to infer a more activated and proliferatively adept state.

Combined Analysis of Pancreatic Tissue and Peripheral Blood

Only one published study has described the functional phenotype of intrapancreatic T-lymphocytes in CP by analysing both pancreas infiltrating and peripheral blood lymphocytes. This seminal work by Schmitz-Winnenthal *et al* was published in *Gastroenterology* in 2010. Isolated lymphocytes from the peripheral blood, bone marrow and tissue of patients with CP, as well as control organ donors without pancreatic disease, were analysed using flow cytometry and enzyme-linked immunosorbent assay (ELISA). They found that Treg cells were significantly expanded in the blood of CP patients compared to controls. There was also a significant enrichment of Treg cells in CP lesions compared to peripheral blood. Furthermore when T-cells isolated from the blood, bone marrow and pancreata of CP patients and healthy donors were cultured with lysates derived from CP tissue, only T-cells from CP patients were stimulated to produce IL-10 (a predominant Treg cell cytokine). They did not however produce the archetypal Th1 and Th2 cytokines IFN-γ and IL-4. These chronic pancreatitis antigen-specific responses were instead mediated by $CD4^{+}CD25^{+}FoxP3^{+}CD127^{-}$ Treg cells.[139]

Interestingly previous authors analysing the peripheral blood of patients with CP found no difference in the proportion of Treg cells in CP patients compared to controls.[140] Nevertheless as Treg cells play a key role in maintaining tolerance to self-antigens, their enrichment in CP tissue may reflect a possible feedback mechanism that attempts to control auto-aggression. Whether this enrichment is mirrored in the peripheral blood of patients might be determined by the activity or severity of the disease. Schmitz-Winnenthal *et al* recruited patients who required surgery and therefore may have had more active or advanced disease than the patients in the other, non-surgical study. It is surprising however that the lymphocytes isolated from CP patients and stimulated with CP antigens did not secrete either IFN-γ or IL-4 indicating polarisation solely to a suppressive and regulatory T-cell phenotypic response.

Pancreatic MHC Expression and Antigenic Targets of Pancreas Infiltrating Lymphocytes

Expression of MHC molecules is a pre-requisite for the development of organ-specific immunity. MHC class I is expressed on the majority of nucleated somatic cells,

however normal exocrine epithelial cells do not express either MHC class I or II molecules.[141, 142] In disease states cellular MHC expression changes and exocrine epithelial cells of chronic pancreatitis patients frequently express β2-microglobulin, a component of MHC I, and HLA-DR, a class II receptor. Simultaneous expression of both MHC class I and II may also be seen in approximately half of CP cases which is not related to the suspected cause of the disease or age.[130, 143]

Initial events during the development of organ specific autoimmunity involve aberrant expression of MHC class II antigens, thus allowing presentation of cell specific antigens to potentially autoreactive T-lymphocytes.[144] Whether tissue damage directly induces MHC expression in pancreatic duct epithelial cells or whether their expression is indirectly stimulated by cytokines such as IFN-γ secreted by infiltrating lymphocytes is unclear.[145] These findings, in association with a known T-lymphocyte infiltrate, establish the possibility that an aberrant cell mediated immune reaction perpetuates pancreatic inflammation resulting in chronic pancreatitis.

The antigens recognised by pancreas infiltrating T-cells are unknown. Autoantibodies reactive to pancreatic acinar cells and ductal antigens have been described in patients with CP, although they were frequently found in controls.[146-148] Activated lymphocytes specifically sensitive to crude pancreatic antigens have also been shown in CP.[149] Furthermore when T-cells isolated from the peripheral blood and bone marrow of CP patients and healthy donors were cultured with lysates derived from CP tissue, only T-cells from CP patients were stimulated to produce cytokines.[139] This supports the notion that T-cells are primed to respond to antigens present in CP lesions and that secondary “autoimmune” mechanisms may participate in the pathogenesis of CP.

Examination of CP tissue for the expression of HLA-DR, Fas receptors (CD95) and its ligand (CD95L) has also produced further evidence for the potential destructive role of T-cells. When CD95 death receptors are oligomerised by the binding of CD95L apoptosis is induced in target cells. Interestingly pancreatic epithelia differentially express CD95 and CD95L in a mutually exclusive manner. In CP the CD95-/CD95L+ status is conserved in islet cells even in the vicinity of lymphocytic infiltrates. This might explain why islets are spared until the late stages of CP as their expression of CD95L promotes peri-islet T-cell apoptosis.[150] Acinar cells that aberrantly co-express HLA-DR lose their CD95-/CD95L+ status. As a potential consequence, and possibly triggered by local release of IFN-γ, which was found to be significantly increased in the CP tissue studied, T-cells may cognately interact with and successfully attack acinar cells by

triggering CD95 on their target without being killed by epithelial, CD95L-mediated, counterattack.[151]

The Interaction of T-Lymphocytes and Pancreatic Stellate Cells

The interaction between pancreatic stellate cells (PSC) and infiltrating T-lymphocytes is likely to be of importance in the perpetuation of fibrosis in the context of ongoing tissue injury. PSC cultured with lymphocytes or PBMC show an increased rate of activation, proliferation and collagen secretion.[152] Specific analysis of such phenomena reveal that IL-13, an archetypal Th2 cell cytokine, promotes proliferation but not activation of cultured rat PSC through suppression of the transcriptional activity of NF-κB resulting in a decrease in autocrine TGF-β1.[153] Conversely IFN-γ inhibits PSC proliferation and activation in cultured rat PSC.[154] These data suggest that pancreatic fibrosis is governed by the same mechanisms that control fibrosis in other tissues; namely that it is driven by Th2 rather than Th1 lymphocytes.

Work by other groups reveals findings that do not fit in with this simplified theory. It has been demonstrated that PSC collagen synthesis is stimulated by both the pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10.[155] IL-10 also contributes to the development of chronic pancreatitis in a coxsackievirus B4-induced mouse model of pancreatitis whereby acute disease resolved when IL-10 was absent or when IL-10 signaling was disrupted.[156] IL-10 is produced by a number of leukocytes but is mainly secreted by monocytes, Th2 and Treg cells. As previously described, lymphocytes isolated from CP tissue and stimulated with CP lysates were frequently found to be Treg cells which produced significant quantities of IL-10.[139] Persistent activation of PSC by cytokines and in particular IL-10 following acute pancreatitis may therefore be a factor involved in the progression from acute pancreatitis to chronic pancreatic injury and fibrosis.

In addition further co-culture studies have demonstrated that PSC influence the lymphocytic infiltrate in CP such that the production of IL-15 by PSC inhibits lymphocyte apoptosis.[152] Further work in hepatic stellate cells (HSC), which show a significant homology to PSC,[157] has demonstrated that co-culture of T-lymphocytes and cytokine stimulated HSC promotes lymphocyte proliferation.[158] This evidence suggests that PSC may play an important role in maintaining a persistent inflammatory infiltrate within the pancreas, thus orchestrating ongoing inflammation and fibrosis.

It is likely that PSC also have a role in antigen presentation. Evidence for this again comes from studies involving PSC and HSC. PSC have been shown to have phagocytic activity.[159] In addition it has been demonstrated that HSC express membrane proteins involved in antigen presentation and T-lymphocyte activation (HLA-I, HLA-II, CD1b CD1c, CD40 and CD80) which are up-regulated following exposure to pro-inflammatory cytokines.[158] This suggests that PSC might be involved in local immune functions in the pancreas by presenting antigen to T-lymphocytes.

The Effects of Alcohol and Smoking on Cellular Immunity, Gut Permeability and Pancreatic Physiology

In the UK, alcohol is implicated in 60 to 70% of cases of chronic pancreatitis, however there is a clear observation that only a minority of heavy drinkers develop pancreatitis.[25] The reason for this remains elusive prompting considerable investigation of susceptibility factors but it is likely that both heavy alcohol consumption in a genetically susceptible host and further triggers are required. Alcohol and its metabolites have a direct toxic effect on acinar cells via production of reactive oxygen species (ROS) which in turn induce autodigestion of acinar cells. This leads to pancreatic necrosis triggering recruitment and activation of inflammatory cells that secrete pro-inflammatory cytokines and chemokines.[160] It is clear that consumption of alcohol alters inflammatory cell responses following both acute and chronic drinking patterns. Numerous studies have demonstrated that chronic alcohol consumption leads to reduced anti-bacterial and anti-viral immunity and more specifically to impaired antigen-specific T-cell activation, alterations in endocytic and phagocytic activity, and defects in antigen presentation.[161-165]

Innate immune responses that are activated by danger signals from pathogens or damaged host tissue are essential for the initiation of appropriate adaptive immune responses. Monocytes, macrophages and dendritic cells that express pattern recognition receptors including toll-like receptors (TLR) comprise elements of the innate immune system that specifically recognise microbial danger signals, such as LPS, a major cell wall component of gram-negative bacteria and TLR4 ligand, and DNA and RNA derived from damaged host cells.[166, 167] Following stimulation and activation of TLR, dendritic cells and monocytes secrete pro-inflammatory cytokines such as TNF-α and IL-1, as well as ROS, which promote T-cell activation. Human monocytes cultured in alcohol and then stimulated with LPS, produce greater amounts of TNF-α and less IL-10 than alcohol-naïve monocytes from the same individual. Additionally they have increased LPS-induced NF-κB binding activity, a transcription factor of pro-

inflammatory cytokine genes.[168] These studies all suggest that amplified pro-inflammatory responses occur after chronic alcohol exposure. Interestingly however a literature search of PubMed (United States National Library of Medicine) using the search terms “alcohol”, “ethanol”, “lymphocyte”, “T-helper” and “regulatory T-cell” found no documented evidence that the proportion or activation of T-lymphocyte subsets are altered by chronic excess alcohol consumption.

In addition to the direct effects of alcohol and its metabolites on acinar cells and leukocytes, altered gut permeability may contribute to pancreatic pathology. Chronic alcohol ingestion causes increased gut permeability leading to bacterial translocation across the mucosal barrier and entry of LPS into the circulation.[169, 170] LPS is known to generate Th1 cell responses which may result in tissue destruction.[171] In rat models of pancreatitis, co-administration of LPS and alcohol resulted in significantly greater pancreatic injury than alcohol alone. If repeated LPS injections were given in the presence of alcohol feeding then pancreatic fibrosis resulted. This is likely to be partly mediated by PSC as they are synergistically activated by LPS and alcohol and they express TLR4.[172] PSC also express TLR9, which recognises bacterial non-methylated cytosine-guanosine dinucleotide (CpG) DNA. PSC migrate and proliferate in response to simulation by CpG DNA suggesting that bacterial DNA can also initiate and sustain pancreatic inflammation and fibrosis.[173]

In another rat model of pancreatitis induced by caerulein, a cholecystokinin analogue that causes dysregulation of digestive enzyme production leading to acinar cell death, alcohol fed rats develop more severe pancreatic injury which results in fibrosis with repeated administration. Initially pro-inflammatory cytokine profiles are elevated however anti-inflammatory cytokines (IL-10 and TGF-β) then become elevated in parallel with fibrogenesis. [174] The findings of these animal models may be related to the fact that pancreatic acini are significantly more sensitive to necrotic cell death following an injurious stimulus such as LPS.[175] This shift from apoptosis to necrosis could cause the potent necro-inflammatory reaction seen in pancreatitis. This would in turn promote infiltration of inflammatory cells and propagate an inflammatory response. As it is known that TNF-α promotes PSC activation it is also conceivable that in the context of chronic alcohol consumption and exposure to LPS, PSC are more potently activated, driving forward the inflammatory response.[155, 176]

Chronic alcohol consumption not only alters intestinal permeability but also promotes the development of small bowel bacterial overgrowth, two features that are likely

linked.[177] Mucosal damage by bacteria and their associated toxins, especially endotoxins from Gram-negative bacteria, may contribute to an enhanced endotoxin translocation from the intestinal lumen into the portal blood. Small bowel bacterial overgrowth is commonly seen in patients with chronic pancreatitis.[178] Furthermore bacterial translocation from the gut is frequently observed in acute pancreatitis and may result in infected pancreatic necrosis. The route of bacterial migration has not yet been clarified. It could be a direct transmural migration to the peritoneal cavity or retroperitoneum and then to the pancreas; or secondary to lymphatic or haematogenous dissemination to the pancreas.[179] There are therefore multiple means by which bacteria or their derived toxins may enter the pancreas and cause injury. It is possible that if the initial pancreatitis event is not complicated by overt infective pancreatic necrosis then low level translocation of bacteria from the gut into the pancreas may promote on-going inflammation, perpetuating the progression from acute to chronic pancreatic inflammatory disease. This inflammatory process could be sustained by T-lymphocytes and in particular may involve MAIT cells that appear to have a role in the detection and destruction of bacterially infected cells.[111, 112] They have not been investigated in pancreatic disease and thus represent an interesting and novel aspect of the immunological response to study.

Cigarette smoking is an independent risk factor for the development of chronic pancreatitis and in combination with alcohol, acts synergistically to accelerate the progression of the disease.[14, 25, 180] It is unclear how smoking exerts its detrimental effect on the pancreas as cigarette smoke contains thousands of constituents, few of which are well-known. However substances such as nicotine, acetaldehyde and cyanamide may alter normal pancreatic exocrine function and modify the concentration of the constituents of pancreatic juice, leading to an increase in the protease to anti-protease ratio. This leads to the generation of pancreatic secretions that are unstable and more likely to become inappropriately activated within the pancreas.[181, 182] Furthermore smoking also leads to the generation of reactive oxygen species that can alter the permeability of cell membranes and promote acinar cell injury.[183] In spite of this nicotine does not affect intestinal barrier function, gastric emptying, small-bowel transit time or orocaecal transit.[184]

In addition to the alterations smoking causes to pancreatic physiology, it can also modify immune and inflammatory responses. Lamina propria T-cells have been shown to significantly up-regulate T-bet but not GATA-3 expression when cultured in nicotine containing medium. This suggests that chronic nicotine stimulation could modulate the

immune balance to a Th1 dominant phenotype.[185] Interestingly however, in experimental models of inflammatory diseases, vagal nerve stimulation by nicotinic agonists, attenuates the production of pro-inflammatory cytokines by immune cells and inhibits inflammatory processes.[186]

It is clear therefore that aberrations in the immune system and gastrointestinal physiology induced by alcohol and smoking likely contribute to the induction and perpetuation of the inflammatory response seen in CP. As no immunological pathway is untouched by alcohol the relationship between CP and immunological dysregulation is clearly complex. The increase in pro-inflammatory cytokine production and potential contributions of LPS, bacterial translocation from the gut and host-derived danger signals may be central to the long-standing T-lymphocyte infiltrate that contributes to on-going inflammation and results in tissue fibrosis. It is important to note that not all cases of CP are associated with either excess alcohol ingestion or smoking and therefore they represent two of a number of possible promoters of the pathogenic pathways. The similar lymphocytic infiltrates seen in CP of different aetiologies does however point to a common pathway of inflammation that once established perpetuates and progresses the disease. Improved understanding of the precise nature of this lymphocytic infiltrate will thus hopefully provide insight into the pathogenesis of CP and potentially aid the future development of novel treatments.

HYPOTHESES

1) The peripheral blood of patients with chronic pancreatitis is comprised of different proportions of T-cell subsets.

2) Specific T-lymphocyte subsets are enriched in chronic pancreatitis tissue.

3) The proportion of T-regulatory cells to T-helper cells is skewed in the pancreatic tissue and peripheral blood of patients with chronic pancreatitis.

4) Mucosal-associated invariant T-cells infiltrate chronically inflamed pancreatic tissue and are accordingly depleted in peripheral blood.

AIMS

1) To develop a reproducible technique to isolate live lymphocytes from pancreatic tissue.

2) Phenotypic analysis of intrapancreatic and peripheral blood lymphocytes in patients with CP using immunohistochemistry, flow cytometry and polymerase chain reaction to identify T-lymphocytes and MAIT cells.

3) Functional characterisation of CD4+ T-helper cell lineages (Th1/2/17) and T-regulatory cells in pancreatic tissue and blood of patients with chronic pancreatitis using flow cytometry.

Chapter 2: Methods

ETHICAL AND RESEARCH AND DEVELOPMENT APPROVAL

This study was conducted at Southampton General Hospital following approval from Oxfordshire Research Ethics Committee A and Southampton University Hospitals NHS Trust Department of Research and Development (REC reference 09/H0604/105; Investigation of the immunological response associated with pancreatic inflammation and cancer development; R&D reference RHM MED 0892).

PATIENT SELECTION

Patient Recruitment

Patients were recruited either at outpatient clinics or at the time of surgical pre-assessment. Informed consent was obtained from all patients (See Appendix – Patient Consent Form and Patient Information Sheet).

Patients were eligible for inclusion in the study:

- If they were due to undergo a surgical operation for benign or malignant disease which involved removal of all or part of the pancreas
- If they had chronic pancreatitis
- If they had no pancreatic disease and were to serve as a healthy control

Peripheral blood and/or pancreatic tissue was collected from patients with the following conditions:

Disease subjects	• Chronic pancreatitis
Control subjects	• Disease necessitating the removal of some, or all, of the pancreas for malignant or non-malignant disease of another organ, which does not involve the pancreas or cause pancreatic duct obstruction e.g. bile duct cancers • Disease within the pancreas which does not affect the surrounding normal tissue e.g. pancreatic neuroendocrine tumours, cystic pancreatic lesions • Healthy individuals without pancreatic disease

The following exclusion criteria applied:

- Any primary disease of the blood or immune system
- Treatment with systemic immunosuppressive drugs of any kind within the previous 5 years (with the exception of courses of corticosteroid therapy of less than 2 weeks duration)
- Acquired immune defects, including human immunodeficiency virus infection
- Any chronic inflammatory disease of the digestive tract or liver e.g. inflammatory bowel disease, cirrhosis
- Systemic chemotherapy, including neoadjuvant chemotherapy at any time in the patient's life
- If the patient is participating in a therapeutic clinical trial
- If the patient has serious difficulty in understanding the purpose and protocol of the study and cannot therefore give informed consent.

Diagnosing Chronic Pancreatitis and Health

The diagnosis of chronic pancreatitis was based on a combination of clinical and radiological criteria:

Clinical Criteria

- Abdominal pain consistent with CP and/or pancreatic steatorrhoea confirmed biochemically by a low faecal elastase level.
- Previous episodes of acute pancreatitis and/or appropriate risk factors for CP e.g. heavy alcohol consumption, smoking and family history.

To substantiate the diagnosis of CP in symptomatic individuals typical pancreatic radiological features shown by cross sectional imaging with either computed tomography (CT) or magnetic resonance imaging (MRI) had to be present. Further imaging with endoscopic retrograde cholangiopancreatography (ERCP) or endoscopic ultrasound (EUS) supported the findings of cross-sectional imaging in some cases.

Radiological Criteria

- Pancreatic calcification
- Duct dilatation
- Parenchymal atrophy
- Presence of complications e.g. pseudocysts

All pancreatic tissue analysed had histological confirmation of normality or chronic pancreatitis as assessed by routine histopathology examination of the non-research tissue resected.

It was ensured that all patients: met the inclusion and exclusion criteria of the research protocol; had a normal full blood count assessed within a week of donating peripheral blood; and had no evidence of autoimmune disease or sepsis. Healthy individuals were additionally screened to confirm that they had no symptoms of pancreatic disease as well as no documented history of pancreatic surgery or acute pancreatitis.

MANIPULATION OF MONONUCELAR CELLS

Venesection

Blood was taken using an aseptic non-touch technique according to accepted protocol. Briefly the skin was cleaned using isopropyl alcohol swabs and blood was taken using a Vacutainer system (BD Bioscience) and collected in tubes containing lithium heparin. Peripheral blood mononuclear cells (PBMC) were isolated shortly after venesection.

Isolation of Peripheral Blood Mononuclear Cells from Whole Blood

It is possible to utilise whole blood for analysing cell surface markers using flow cytometry. However it is advantageous when attempting to identify rarer epitopes to have a more condensed pool of cells. I therefore chose to isolate peripheral blood mononuclear cells from whole blood taken by venesection.

The isolation of PBMC, comprising lymphocytes and monocytes, by density gradient centrifugation utilises differences in cellular density to allow separation of mononuclear cells from granulocytes and erythrocytes. When blood is layered over Ficoll-Paque Plus and centrifuged, a layer of mononuclear cells rests at the interface between the plasma and Ficoll-Paque Plus, whilst heavier granulocytes and erythrocytes move into the lower layer. Mononuclear cells can then be recovered from the interface.

Method

Whole blood was mixed with equal volumes of phosphate buffered saline (PBS) without calcium and magnesium (Lonza) and then layered onto Ficoll-Paque Plus (GE Healthcare) before centrifuging at 2000 rpm at room temperature for 20 minutes. PBMC were harvested from the interface and washed with PBS by centrifuging at 1500 rpm at room temperature for 15 minutes. The pellet was re-suspended in cold PBS and

cells were counted using a haemocytometer and viability determined by trypan blue (Lonza) exclusion, before centrifuging again at 1200 rpm at 4°C for 12 minutes. The pellet was then suitable for dividing and storage using cryopreservation.

Cryopreservation of Mononuclear Cells

I planned to undertake a number of different experiments on each patient's donated samples. As it was not technically feasible to perform all of the experiments immediately subsequent to the isolation of PBMC or mononuclear cells from pancreatic tissue I elected to cryopreserve all harvested cells.

Method

Isolated PBMC/mononuclear cells were frozen using a freezing medium consisting of 90% foetal calf serum (FCS) (Invitrogen) and 10% dimethyl sulfoxide (DMSO) (Sigma). DMSO serves as a cryoprotectant, reducing ice formation during the freezing process. After counting, cells were re-suspended in the freezing medium at a concentration of 5 - 10 x 10^6 cells/ml and then aliquoted into 1ml cryovials, which were cooled on ice. The mononuclear cells were allowed to equilibrate with the freezing media for 5 minutes before being placed into a Mr Frosty Cryo 5100 slow-freeze container (Nalgene) containing 100% isopropyl alcohol (Sigma) which was then placed in a -80°C freezer for 24 hours. The cryovials were finally transferred into a liquid nitrogen containing Dewar for long term storage.

Thawing of Mononuclear Cells

Cryovials were removed from the liquid nitrogen storage Dewar and placed in a water bath at 37°C. As soon as the cell suspension had thawed it was transferred to a 15ml falcon tube and warm RPMI Wash Solution (See Appendix) was added drop by drop until a total volume of 10ml was reached. The cells were washed by centrifuging at 1500rpm for 5 minutes at room temperature. The pellet was then re-suspended in warm RPMI Wash Solution and the cells counted using a haemocytometer with viability determined by trypan blue exclusion. The cells were washed again by centrifuging at 1500rpm for 5 minutes at room temperature before the resulting pellet was re-suspended in media suitable for carrying out flow cytometry (PBS) or cell stimulation. Mononuclear cells intended for stimulation were suspended in RPMI Lymphocyte Culture Media (See Appendix) and placed in flat bottomed 48 well plates at a concentration of 0.5 x 10^6 cells/ml, 0.5ml/well, and incubated overnight at 37°C and 5% CO_2.[187]

Stimulation of Mononuclear Cells

Quiescent lymphocytes secrete cytokines in quantities below the detection limit of conventional assays. It is therefore standard practice to stimulate cells in vitro prior to performing cytokine assays. Phorbol 12-myristate 13-acetate (PMA) is a potent lymphocyte mitogen which activates the signal transduction enzyme protein kinase C (PKC). When lymphocytes are cultured in media containing PMA and ionomycin, a calcium ionophore which synergises with PMA in enhancing PKC activation, it results in intracellular cytokine production. The carboxylic ionophore monensin is also used to interrupt intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex, improving detection sensitivity.[188] T-helper cell subsets may then be identified using intracellular cytokine staining and flow cytometry analysis.[188-191] Whilst several other techniques exist to stimulate T-cells, the use of PMA and ionomycin has been shown to be the most effective means of inducing cytokine production *in vitro* whilst maintaining cell viability and function.[192] PMA and ionomycin stimulation however does have the deleterious effect of altering T-cell morphology and down regulating CD4 and CD8 membrane expression. Despite this, clearly identifiable CD4+ and CD8+ T-cell subsets may still be identified.[188, 192, 193]

Method

Mononuclear cells were stimulated with PMA (Sigma) (at final concentration of 25ng/ml) and ionomycin (Sigma) (at final concentration of 500ng/ml). In addition monensin (eBioscience) (at a final concentration of 2μM) was added to inhibit intracellular protein transport. The cells were then incubated at 37°C and 5% CO_2 for five hours.

ISOLATING LYMPHOCYTES FROM PANCREATIC TISSUE

Analysis of pancreas infiltrating lymphocytes has most frequently been undertaken using immunohistochemistry. At the time I began this research only one group had previously reported the isolation of lymphocytes from human chronic pancreatitis tissue, therefore a standard, well-established and widely reproduced protocol was not available. One of the principle initial aims of this project therefore was to develop a new technique to isolate lymphocytes from pancreatic tissue. The following methods describe in detail the laboratory processes undertaken. An explanation of the rationale and planning, along with the results of all optimisation experiments, are shown in Chapter 3.

Collection and Transportation of Pancreatic Tissue

All protocols developed followed the same initial few steps: The pancreatic tissue was collected directly from the operating theatre and immediately upon its removal placed into ice cold Hanks Balanced Salt Solution containing calcium (HBSS(+)). It was then transported back to the laboratory on ice, a journey taking only 2-3 minutes, and weighed. If two methodologies were being compared then it was cut in half before weighing.

Protocol 1

This initial approach aimed to validate whether mechanical digestion alone or mechanical plus enzymatic digestion gave superior yields of lymphocytes:

Method A

The pancreatic tissue was minced finely using scissors and then pushed through a Falcon cell strainer. The cellular suspension was then washed with ice cold Lymphocyte Isolation Buffer (See Chapter 3 and Appendix) and centrifuged at 400rpm for 3 minutes at 4°C. Both the supernatant and pellet were collected:

- The supernatant was centrifuged at 1500rpm for 5 minutes at 4°C and the resulting pellet was washed in ice cold HBSS(+) and centrifuged at 1500rpm for 5 minutes at 4°C.
- The pellet was re-suspended in ice cold Lymphocyte Isolation Buffer and centrifuged at 1500rpm for 5 minutes at 4°C. The pellet formed was then washed in ice cold HBSS(+) and centrifuged at 1500rpm for 5 minutes at 4°C.

Method B

The pancreatic tissue was minced finely using scissors, added to Collagenase II (1mg/ml) made up in Complete Medium (See Appendix), and placed in a shaking incubator at 37°C for 30 minutes. The resulting cellular suspension was then pushed through a Falcon cell strainer, adding DNase (1mg/ml) (Roche) made up in Complete Medium, to wash cells through and prevent clumping. The suspension was subsequently washed with ice cold Lymphocyte Isolation Buffer and centrifuged at 400rpm for 3 minutes at 4ºC. Both the supernatant and pellet were collected:

- The supernatant was centrifuged at 1500rpm for 5 minutes at 4°C and the resulting pellet was washed in ice cold Complete Medium and centrifuged at 1500rpm for 5 minutes at 4°C.
- The pellet was re-suspended in ice cold Lymphocyte Isolation Buffer and centrifuged at 1500rpm for 5 minutes at 4°C. The pellet formed was then washed in ice cold Complete Medium and centrifuged at 1500rpm for 5 minutes at 4°C.

The pellets resulting from both methods were then individually re-suspended in 38% Percoll (Sigma) made up with PBS, underlaid with 73% Percoll made up with HBSS(-), overlaid with 19% Percoll made up with HBSS(-), and centrifuged at 900rpm for 15 minutes at room temperature. Cells in the lower interphase were collected and then washed with Complete Medium and centrifuged at 1500rpm for 5 minutes at room temperature. The pellet was re-suspended in HBSS(+) and lymphocytes were counted using a haemocytometer and viability determined by trypan blue exclusion. The suspension was then centrifuged at 1500rpm for 5 minutes at 4°C to form a pellet which was re-suspended either in PBS, for assessment using flow cytometry, or in 90% foetal calf serum and 10% DMSO, for cryopreservation.

Method B was found to yield the highest numbers of lymphocytes (See Chapter 3).

Protocol 2

The second approach aimed to establish the optimum working concentration of Collagenase II enzyme. Method B was followed, however the minced pancreatic tissue was placed in Complete Medium containing Collagenase II at either 1mg/ml or 2mg/ml. A further change to Method B was the use of more density gradient layers, whereby the four cell pellets were re-suspended in 40% Percoll made up with PBS, and then layered into 15 ml Falcon tubes (See Figure 7) and centrifuged at 900rpm for 15 minutes at room temperature.

Figure 7: Density Gradient Centrifugation of Pancreatic Tissue Cell Suspensions

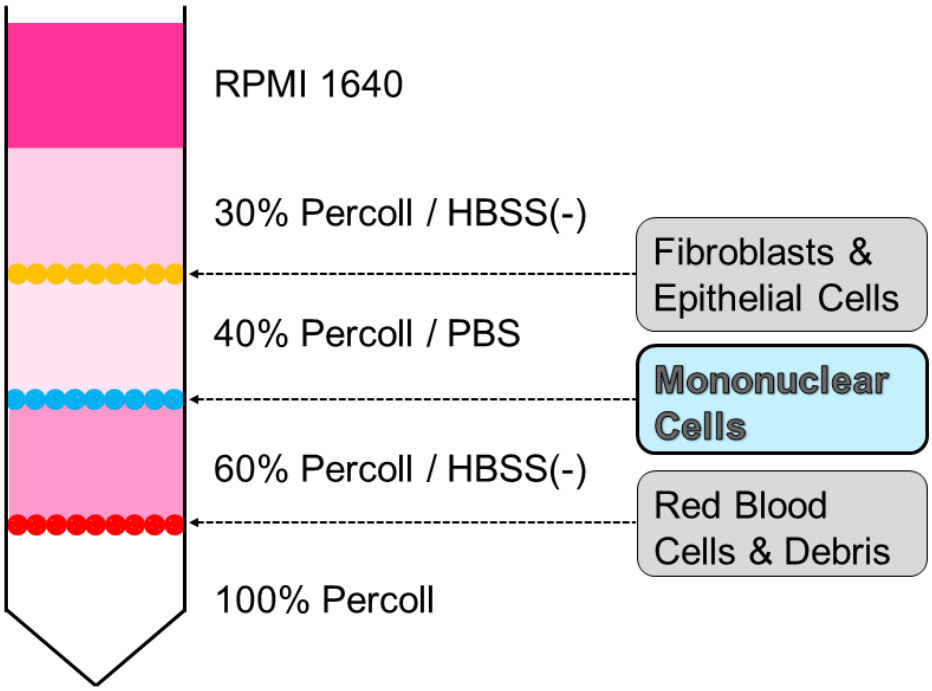


Figure 7 Legend

To separate cells in pancreatic tissue homogenates using density gradient centrifugation, solutions of different densities were layered on top of each other in a 15ml Falcon tube. The pancreatic tissue cell suspension was initially within the 40% Percoll layer, however following centrifugation at 900rpm for 15 minutes at room temperature, mononuclear cells could be harvested from the interface between the 40% and 60% Percoll layers.

Protocol 3

The initial centrifugation step at 400rpm was implemented to promote removal of cellular debris, however I frequently found appreciable numbers of lymphocytes within the pellet obtained. As the method for density gradient centrifugation had been augmented and allowed cell debris to be more effectively isolated I increased the speed and duration of the initial centrifugation step to 1500rpm for 5 minutes at 4°C (as per isolation of PBMC). The supernatant was then centrifuged again at 1500rpm for 5 minutes at 4°C to ensure optimal lymphocyte collection and the pellets re-suspended and combined.

The Definitive Protocol

There was little difference between using Collagenase II at 1mg/ml and 2mg/ml (See Chapter 3). Therefore I opted to use 1mg/ml for all further experiments. The definitive protocol therefore was:

The pancreatic tissue was minced finely using scissors, added to Collagenase II (1mg/ml) made up in Complete Medium, and placed in a shaking incubator at 37°C for 30 minutes. The resulting cellular suspension was then pushed through a Falcon cell strainer, adding DNase (1mg/ml) made up in Complete Medium, to wash cells through. The suspension was subsequently washed with ice cold Lymphocyte Isolation Buffer and centrifuged at 1500rpm for 5 minutes at 4ºC. Both the supernatant and pellet were collected:

- The supernatant was centrifuged at 1500rpm for 5 minutes at 4°C and the resulting pellet was washed in ice cold Complete Medium and centrifuged again at 1500rpm for 5 minutes at 4°C.
- The pellet was re-suspended in ice cold Lymphocyte Isolation Buffer and centrifuged at 1500rpm for 5 minutes at 4°C. The pellet formed was then washed in ice cold Complete Medium and centrifuged at 1500rpm for 5 minutes at 4°C.

The two resulting pellets were combined and re-suspended in 40% Percoll made up with PBS, layered into 15 ml Falcon tubes (See Figure 7) and centrifuged at 900rpm for 15 minutes at room temperature. Cells in the lower middle interphase were collected, washed with RPMI Wash Solution and centrifuged at 1500rpm for 5 minutes at room temperature. The resulting pellet was then re-suspended again in RPMI Wash Solution and lymphocytes were counted using a haemocytometer and viability determined by trypan blue exclusion. The suspension was finally centrifuged at 1500rpm for 5 minutes at 4°C to form a pellet which was re-suspended in in 90% foetal calf serum and 10% DMSO, for cryopreservation.

FLOW CYTOMETRY

The essential principle of flow cytometry is that single cells or particles suspended within a laminar flow of liquid are interrogated individually to measure their optical and fluorescent characteristics as they pass through a focused light source; in this case three lasers which have different excitation wavelengths. As the cells of interest intercept the light sources they scatter light, giving a measure of the physical properties of the cell such as size (shown by forward angle scatter) and internal complexity (shown by side angle scatter). Fluorochromes, which may be directly or indirectly conjugated to antibodies that bind specific proteins on the surface of or within cells, are also excited to a higher energy state causing the release of energy as a photon of light with specific spectral properties unique to it. Optical detectors subsequently direct scattered and emitted light into a series of filters and mirrors to isolate particular wavelengths. The light signals are then detected and digitised for computer analysis.

Equipment Setup

Cell suspensions were analysed using a FACSAria II flow cytometer (BD Bioscience) running FACSDiva software (BD Bioscience). A minimum of 20,000 events were recorded from each sample. Data was subsequently analysed using FlowJo software (Tree Star). Cell populations were “gated” according to a step-wise hierarchy that allowed the identification of single viable cells of interest within a heterogeneous population. The initial gate was set to identify lymphocytes using their forward and side scatter characteristics. Live cells were then identified, followed by CD3+ T-cells. Further analysis of T-cell subsets and lineages was subsequently undertaken whereby the creation of gates to identify negative, unstained cells using isotype controls, allowed the detection of positively stained cells (e.g. Figure 12: PBMC Analysis Using Flow Cytometry). In each results section the gating strategy employed will be precisely defined in terms of each T-cell population.

To correct for spectral overlap during multicolor flow cytometry experiments, colour compensation must be performed. The goal of colour compensation is to correctly quantify each fluorescent dye with which a particular cell is labeled. This is done by subtracting a portion of one detector's signal from another, leaving only the desired signal. This process was achieved by running samples that were individually stained with each fluorochrome of the complete multi-colour panel desired. Rather than using all of the antibodies intended, I used fluorochromes conjugated to antibodies that recognise common epitopes (e.g. CD3), rather than rarer ones (e.g. Integrin α4). By

selecting clear positive stained cells and using FACSDiva automatic compensation setup, a working protocol was established which could be used for all subsequent analyses. Compensation setup was repeated a number of times during the project, particularly following servicing and repairs to the FACSAria which resulted in a change of a laser's intensity. The optimal working dilution for each antibody was also assessed and repeated for each new batch of antibody.

Creating Panels of Antibodies

To identify as many lymphocyte subsets and lineages as possible using the fewest number of reagents would allow the generation of large quantities of data and be fiscally prudent. As fluorochromes exhibit a degree of spectral overlap it was necessary to match the capabilities of our FACSAria with appropriate antibody-fluorochrome combinations. These are shown in Table 1, Table 2, Table 28 and Table 29.

Controls

There is considerable debate about the question of appropriate negative controls when staining cells with a direct protocol that uses fluorochrome conjugated antibodies. There are three possible controls that may be used:

i) If positively stained cells are clearly separated from unstained, auto-fluorescent cells, and if the experiment contains samples where no cells stain, then arguably the unstained cells within the stained samples are in themselves controls for non-specific staining.
ii) When intracellular cytokines are to be detected using flow cytometry following stimulation with PMA and ionomycin, a cell population which is stained but which has not been stimulated, serves as an appropriate negative control.
iii) Isotype control antibodies may be used. These are antibodies of the same immunoglobulin sub-type, which do not specifically bind to antigens of interest, and are conjugated to the same fluorochrome. Non-specific binding to cells will therefore produce positive fluorescence shift.

The use of isotype control antibodies has significant limitations. Although they should be matched to the immunoglobulin sub-type, used at the same protein concentration, and conjugated to the same fluorochrome as the test antibody, they have inherently different antigen specificities to the test antibody as the tertiary structures are non-identical. Furthermore as the exact protein concentration of the antibody and the number of fluorochrome molecules conjugated to each of the antibodies is unknown, the degree of fluorescence added to the negative cells by nonspecific binding may not

be identical to that of the test antibody. The use of isotype control antibodies can consequently be misleading. Nevertheless isotype controls provide a useful, if imperfect, negative control for flow cytometry analysis and help define a clear starting point for identifying positive staining. I therefore chose to use all three techniques, however for large and clearly visible cell populations, e.g. CD3+ T-cells, I stopped using isotype control antibodies after a few experiments.

Table 1: Antibodies and Dyes Used for Flow Cytometry

Fluorochrome	Panel 1	Panel 2	Panel 3	Panel 4
	Quantification of Lymphocytes	Phenotype of T-Cell Lineages	Quantification of Treg Cells	MAIT Cells
FITC	CD161	IL-13	CD127	CD161
PE	Integrin α4	IL-17	CD25	TCR Vα7.2*
PE-TR	CD3	CD3	CD3	CD3
PerCP-Cy5.5	CD4	CD4	CD4	CD4
PE-Cy7	CD45RO	IFN-γ	---	---
APC	Integrin β7	---	FoxP3	---
APC-Cy7	CD8	CD8	---	CD8
Violet	Live/Dead Fixable Dye	Live/Dead Fixable Dye	Live/Dead Fixable Dye	Live/Dead Fixable Dye

Table 1 Legend

*FITC - Fluorescein Isothiocyanate; PE - Phycoerythrin; PE-TR – Phycoerythrin-Texas Red; PerCP-Cy5.5 - Peridinin chlorophyll protein-Cyanine Dye 5.5; PE-Cy7 – Phycoerythrin-Cyanine Dye 7; APC - Allophycocyanin; APC-Cy7 – Allophycocyanin-Cyanine Dye 7. *Biotinylated antibody identified using streptavidin conjugated to PE.*

Table 2: Antibody Combinations Allowing Identification of Different Cell Types

	Cells Identified	Cellular Markers
Panel 1	T-Cells	$CD3^+$
	T-Helper Cells	$CD3^+$ $CD4^+$
	T-Cytotoxic Cells	$CD3^+$ $CD8^+$ ($CD161^+$)
	Double Negative T-Cells	$CD3^+$ $CD4^-$ $CD8^-$
	Memory T-Cells	$CD3^+$ $CD4^+$ or $CD8^+$ $CD45RO^+$
	Naïve T-Cells	$CD3^+$ $CD4^+$ or $CD8^+$ $CD45RO^-$
	Gut Homing T-Cells	$CD3^+$ $CD4^+$ or $CD8^+$ Integrin $\alpha4^+\beta7^+$
Panel 2	Th1 Cells	$CD3^+$ $CD4^+$ IFN-γ^+
	Th2 Cells	$CD3^+$ $CD4^+$ IL-13^+
	Th17 Cells	$CD3^+$ $CD4^+$ IL-17^+ (IFN-γ^+)
Panel 3	Treg Cells	$CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ $CD127^{-/low}$
	Activated T-Helper Cells	$CD3^+$ $CD4^+$ $CD25^+$
Panel 4	MAIT Cells	$CD3^+$ $CD4^-CD8^-$ or $CD4^-CD8^+$ $CD161^+$ TCR $V\alpha7.2^+$

Table 2 Legend

Panels of antibodies are referred to in Table 1.

Identifying Cell Surface Antigens: Direct Staining Method

Mononuclear cells were re-suspended in cold PBS to a final concentration of 20 x 10^6/ml. 500,000 cells were placed into each FACS tube and dye/antibody/isotype cocktails were added as per Table 3. Following addition of the cocktails, the tubes were gently agitated, covered and incubated on ice for 30 minutes. Each sample was then washed with 3ml PBS and centrifuged at 1500rpm for 5 minutes at 4ºC. The resulting pellet was fixed by re-suspending the cells in 10% neutral buffered formalin (Sigma) and incubating on ice for 15 minutes. A final wash was undertaken before re-suspension in FACS buffer (See Appendix). The sample was then covered and stored at 4ºC until analysis (within 24 hours).

Table 3: Hierarchy of Staining to Directly Identify Cell Surface Antigens

Tube Name	Dye / Antibody / Isotype Cocktail	
Unstained	No antibodies added	
Isotype 1	Live/Dead Fixable Violet Dye	IgG2a PE-TR (Isotype to anti-CD3)
Isotype 2	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	IgG1κ PerCP-Cy5.5 (Isotype to anti-CD4)	IgG1κ APC-Cy7 (Isotype to anti-CD8)
Isotype 3	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	anti-CD4 PerCP-Cy5.5	anti-CD8 APC-Cy7
	IgG1κ FITC (Isotype to anti-CD161)	IgG1 PE (Isotype to anti-Integrin α4)
	IgG2a APC (Isotype to anti-Integrin β7)	IgG2a PE-Cy7 (Isotype to anti-CD45RO)
Stained	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	anti-CD4 PerCP-Cy5.5	anti-CD8 APC-Cy7
	anti-CD161 FITC	anti-Integrin α4 PE
	anti-Integrin β7 APC	anti-CD45RO PE-Cy7

Identifying Cell Surface Antigens and Intracellular Cytokines

Cells were initially stimulated as previously described or left unstimulated to serve as negative controls (See Table 4). All cells were then washed with 3ml PBS at room temperature and centrifuged at 1500rpm for 5 minutes at 21°C. The resulting pellets (except unstained tube) were subsequently re-suspended with a live/dead fixable violet dye (Invitrogen) in PBS, gently agitated, covered and incubated on ice for 30 minutes. Following incubation each sample was washed with PBS before addition of Fixation-Permeabilisation buffer (eBioscience) and incubation on ice for 45 minutes. The cells were then washed twice with Permeabilisation buffer (eBioscience) and centrifuged at 1500rpm for 5 minutes at 4°C. The extra-cellular antibody/isotype cocktails (See Table 4 – unshaded boxes), made up in Permeabilisation buffer, were then added, the tubes gently agitated, covered and incubated on ice for 15 minutes. The intra-cellular antibody/isotype cocktails (See Table 4 – shaded boxes), made up in Permeabilisation buffer, were then added, and the tubes gently agitated, covered and incubated on ice for 30 minutes. Following incubation each sample was washed twice with Permeabilisation buffer before re-suspension in FACS buffer. The sample was then covered and stored at 4ºC until analysis (within 24 hours).

Table 4: Hierarchy of Staining to Identify Cell Surface Antigens and Intracellular Cytokines

Tube Name	Antibody / Isotype Cocktail	
Unstained	No antibodies added	
Isotype 1	IgG2a PE-TR (Isotype to anti-CD3)	----------
Isotype 2	anti-CD3 PE-TR	IgG1κ PerCP-Cy5.5 (Isotype to anti-CD4)
	IgG1κ APC-Cy7 (Isotype to anti-CD8)	----------
Isotype 3	anti-CD3 PE-TR	anti-CD4 PerCP-Cy5.5
	anti-CD8 APC-Cy7	----------
	IgG1κ FITC (Isotype to anti-IL-13)	IgG1 PE (Isotype to anti-IL-17)
	IgG1 PE-Cy7 (Isotype to anti-IFN-γ)	----------
Stained Unstimulated / Stained Stimulated	anti-CD3 PE-TR	anti-CD4 PerCP-Cy5.5
	anti-CD8 APC-Cy7	----------
	anti-IL-13 FITC	anti-IL-17 PE
	anti-IFN-γ PE-Cy7	----------

Table 4 Legend

Highlighted/shaded boxes indicate antibodies to intracellular antigens.

Identifying Cell Surface Antigens and Transcription Factors

Mononuclear cells were re-suspended in cold PBS to a final concentration of 20 x 10^6/ml. 500,000 cells were placed into each FACS tube and extracellular dye/antibody/isotype cocktails were added (See Table 5 – unshaded boxes). Following addition of the cocktails, the tubes were gently agitated, covered and incubated on ice for 30 minutes. Each sample was then washed with 3ml PBS and centrifuged at 1500rpm for 5 minutes at 4ºC. Samples were then re-suspended in Fixation-Permeabilisation buffer and incubated on ice for 45 minutes. The cells were subsequently washed twice with Permeabilisation buffer and then the unstained, isotype 1 and isotype 2 tubes were re-suspended in Permeabilisation buffer; and the isotype 3 and stained tubes in 2% rat serum (eBioscience) made up in Permeabilisation buffer. The cells were then incubated on ice for 15 minutes before addition of IgG2a APC to the isotype 3 tube and anti-Foxp3 APC to the stained tube (See Table 5 – shaded boxes). The cells were gently agitated, covered and incubated on ice for 45 minutes. Following incubation each sample was washed twice with Permeabilisation buffer before re-suspension in FACS buffer. The sample was then covered and stored at 4ºC until analysis (within 24 hours).

Table 5: Hierarchy of Staining to Identify Cell Surface Antigens and Transcription Factors

Tube Name	Dye / Antibody / Isotype Cocktail	
Unstained	No antibodies added	
Isotype 1	Live/Dead Fixable Violet Dye	IgG2a PE-TR (Isotype to anti-CD3)
Isotype 2	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	IgG1κ PerCP-Cy5.5 (Isotype to anti-CD4)	
Isotype 3	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	anti-CD4 PerCP-Cy5.5	IgG1κ FITC (Isotype to anti-CD127)
	IgG1 PE (Isotype to anti-CD25)	IgG2a APC (Isotype to anti-FoxP3)
Stained	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	anti-CD4 PerCP-Cy5.5	anti-CD127 FITC
	anti-CD25 PE	anti-FoxP3 APC

Table 5 Legend

Highlighted/shaded boxes indicate antibodies to intracellular antigens.

Identifying Cell Surface Antigens: Direct and Indirect Staining Method

Mononuclear cells were re-suspended in cold PBS to a final concentration of 20 x 10^6/ml. 500,000 cells were placed into each FACS tube and dye/antibody/isotype cocktails were added as per Table 6. Following addition of the cocktails, the tubes were gently agitated, covered and incubated on ice for 30 minutes. Each sample was then washed with 3ml PBS and centrifuged at 1500rpm for 5 minutes at 4ºC. The unstained, isotype 1 and isotype 2 tubes were subsequently re-suspended in FACS buffer, and the isotype 3 and stained tubes were re-suspended with streptavidin-PE (BD Bioscience) in PBS. The tubes were gently agitated, covered and incubated on ice for 30 minutes. The samples were then washed again before fixing with 10% neutral buffered formalin and incubating on ice for 15 minutes. A final wash was undertaken before re-suspension in FACS buffer. The sample was then covered and stored at 4ºC until analysis (within 24 hours).

Table 6: Hierarchy of Staining to Directly and Indirectly Identify Cell Surface Antigens

Tube Name	Dye / Antibody / Isotype Cocktail	
Unstained	No antibodies added	
Isotype 1	Live/Dead Fixable Violet Dye	IgG2a PE-TR (Isotype to anti-CD3)
Isotype 2	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	IgG1κ PerCP-Cy5.5 (Isotype to anti-CD4)	IgG1κ APC-Cy7 (Isotype to anti-CD8)
Isotype 3	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	anti-CD4 PerCP-Cy5.5	anti-CD8 APC-Cy7
	IgG1κ FITC (Isotype to anti-CD161)	See note below†
Stained	Live/Dead Fixable Violet Dye	anti-CD3 PE-TR
	anti-CD4 PerCP-Cy5.5	anti-CD8 APC-Cy7
	anti-CD161 FITC	anti-TCR Vα7.2

† The control was addition of streptavidin-PE to isotype 3 tube without prior incubation with anti-TCR Vα7.2

Fluorescence-Activated Cell Sorting

It is possible to isolate individual cells using flow cytometry. The cell suspension is entrained in the centre of a narrow, rapidly flowing stream of liquid. The stream is broken into individual droplets by a vibrating mechanism which is adjusted so that there is a low probability that each droplet encapsulates more than one cell. Prior to the stream breaking into droplets, the flow passes through fluorescence detectors which determine the characteristics of interest of each cell. An electrical charge is then placed on the droplet and the charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge.

Fluorescence-activated cells sorting (FACS) was employed to isolate $CD3^{+}V\alpha7.2^{-}$, $CD3^{+}V\alpha7.2^{+}$, $CD3^{+}CD4^{-}CD8^{+}V\alpha7.2^{+}CD161^{+}$, $CD3^{+}CD4^{-}CD8^{+}V\alpha7.2^{+}CD161^{-}$, $CD3^{+}CD4^{-}CD8^{-}V\alpha7.2^{+}CD161^{+}$, and $CD3^{+}CD4^{-}CD8^{-}V\alpha7.2^{+}CD161^{-}$ T-cells from PBMC and pancreatic mononuclear cell suspensions. The staining protocol used was identical to that used for identifying cell surface antigens: direct and indirect staining method, with the exception that the cells were not fixed with neutral buffered formalin. Cells were sorted into lysis buffer containing β-Mercaptoethanol (Stratagene Absolutely RNA Nanoprep Kit, Agilent Technologies), vortexed for 30 seconds, centrifuged at 10,000rpm for 2 minutes at room temperature and then placed in a -80°C freezer for storage.

EXTRACTION AND PREPARATION OF RNA

RNA extraction allows the isolation and purification of RNA from biological samples to allow their use in further analysis e.g. for polymerase chain reaction.

Method

RNA was extracted from lysed cells acquired by FACS using the Stratagene Absolutely RNA Nanoprep Kit (Agilent Technologies). This method has been previously shown in our laboratory to be a sensitive and reproducible technique for extracting RNA from low cell numbers e.g. fewer than 10,000 cells.[194] Appropriate precautions were taken to avoid RNA degradation and contamination, such as use of RNase-free ethanol and water (Ambion), sterile filter tips, and cleaning equipment with RNase Away (Fisher Scientific). Given the low cell numbers from which the RNA was isolated, often less than 2,000 cells, RNA quantitation was not undertaken as this would have utilised a small but significant proportion of the total sample.

REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION

Reverse transcription creates cDNA from an RNA template. A specific sequence within the cDNA may then be exponentially amplified using polymerase chain reaction. This relies on repeated thermal cycling, whereby repeated heating and cooling of the reaction allows for DNA denaturation, annealing and extension with enzymatic replication of the DNA. Primers containing DNA sequences complementary to the target region, along with a heat stable DNA polymerase, enable this selective and repeated amplification. The PCR product may then be identified using Agarose gel electrophoresis, which allows for the estimation of the size of DNA fragments generated.

Primers

The primers used were designed by Professor Stephan Gadola and Dr. Salah Mansour (postdoctoral researcher). The full nucleotide sequence of the MAIT TCR is shown in Figure 8, along with the forward and reverse primers used. The PCR product of the Vα7.2-Jα33 MAIT TCR sequence is 376 nucelotides long.

Figure 8: Full Human MAIT TCR Nucelotide Sequence and Primers used for PCR

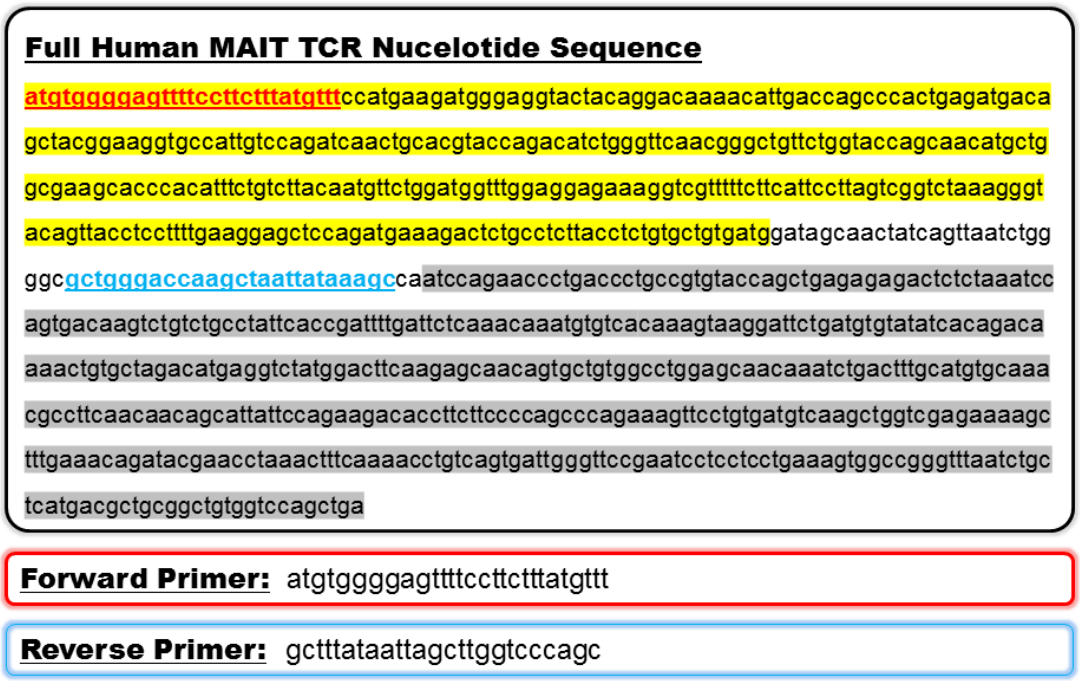


Figure 8 Legend

Full human MAIT TCR sequence: Text in red and blue represents targets for forward and reverse primers respectively; TCR Vα7.2 region highlighted yellow; TCR Jα33 region without highlight; TCR constant region highlighted grey.

Reverse Transcription

First strand cDNA was made from RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Reverse transcription was conducted in 20µl aliquots comprising 10µl RT Buffer, 1µl RT Enzyme Mix and 9µl RNA sample; and incubated at 37°C for 60 minutes using a MJ Research DNA Engine Tetrad 2 Peltier Thermal Cycler. The reaction was then stopped by heating to 95°C for 5 minutes before cooling to 4°C. Negative controls included with each experiment were samples containing either no RNA or no reverse transcriptase enzyme.

Polymerase Chain Reaction

PCR was undertaken using the Phusion High-Fidelity PCR Kit (New England Biolabs). Each 20µl reaction comprised: 4µl GC buffer, 0.4µl deoxynucleotide solution mix (10mM), 0.6µl DMSO and 0.2µl Phusion hot start DNA polymerase enzyme; plus 2µl each of forward and reverse primers (4mM) (Eurofins MWG Operon); plus 3µl betaine (0.8M) (Sigma) and 6.8µl RNase-free water (Ambion) to reduce the formation of

secondary structures in the GC-rich regions. The reaction was initially denatured by incubating at 98°C for 30 seconds; followed by 40 cycles of:

- 10 seconds of denaturing at 98°C
- 30 seconds of annealing at 65°C
- 30 seconds of extension at 72°C

This was then concluded with 5 minutes of final extension at 72°C and then cooling to 4°C; using a MJ Research DNA Engine Tetrad 2 Peltier Thermal Cycler. Negative controls included with each experiment were samples containing either no cDNA or no DNA polymerase enzyme.

AGAROSE GEL ELECTROPHORESIS

An agarose gel was made by dissolving electrophoresis grade Agarose (1%) (Sigma) and SYBR Safe DNA Stain (1:10,000) (Invitrogen) in Tris/Borate/EDTA solution (TBE) (Sigma) which was poured into an electrophoresis mould containing a comb. When the gel had set it was covered with TBE and the gel's lanes were loaded with either the PCR product mixed with a loading buffer or a ladder (SmartLadder Short Fragment MW-1800-04 (Eurogentec). The gel was run at 100 volts for one hour and subsequently viewed using ultraviolet light.

IMMUNOHISTOCHEMISTRY

This technique allows the identification of specific antigens in tissues using antibodies which are linked to visible markers. Indirect immunohistochemistry employs a three stage process to allow the visible signal to be significantly amplified: a primary antibody is used to detect the antigen; a biotinylated secondary antibody then binds the Fc segment of the primary antibody; and an avidin-biotin enzyme complex binds the secondary antibody which is finally detected using streptavidin peroxidise conjugated with a chromagen (See Figure 9).

Figure 9: Indirect Immunohistochemistry Staining Procedure

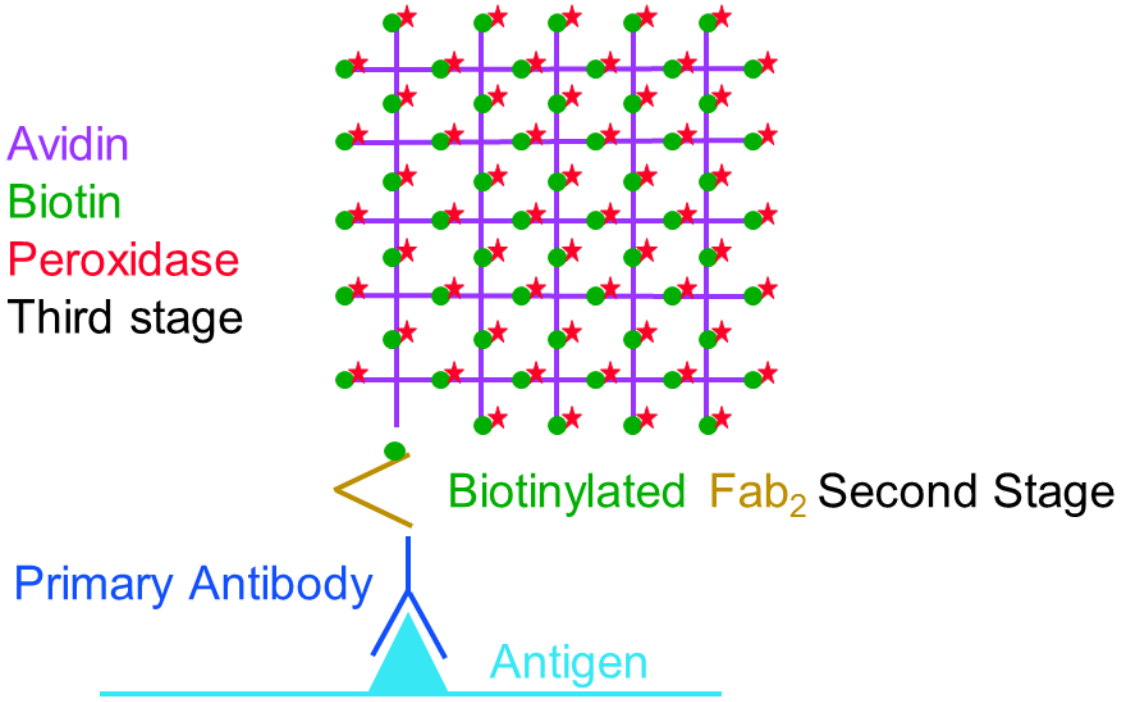


Figure 9 Legend

The indirect immunohistochemistry staining method utilises a primary antibody to identify a specific antigen. A biotinylated secondary antibody then binds to the Fab fragment of the primary antibody. An avidin-biotin-peroxidase third stage binds to the secondary antibody and significantly amplifies the colour signal which develops following the addition of a chromagen e.g. AEC.

Rationale for Methods Employed

I aimed to identify the surface antigens and intracellular transcription factors and cytokines of T-lymphocytes in pancreatic tissue using antibodies that would complement those used for flow cytometry. I therefore chose to identify: CD3, CD4,

CD8, FoxP3, IFN-γ, IL-13 and IL-17. As a large paraffin embedded tissue archive is available at Southampton General Hospital I initially aspired to use these specimens, however antibodies that have been tested and are suitable for such an application were not commercially available. I consequently gained samples of the potential antibodies and tested them on human paraffin embedded tonsil and jejunal tissue using a variety of techniques to unmask and retrieve antigens cross-linked by their fixation and processing into paraffin, including: Microwave or pressure cooker heat mediated pre-treatment using citric acid or EDTA buffer; and Proteolytic pre-treatment using pronase solution. Unfortunately reproducible staining of the complete repertoire of antigens was not possible and therefore this approach was abandoned (See Table 30: Antibodies Tested for Paraffin Immunohistochemistry in Appendix). I therefore began to collect fresh tissue and process it into glycol methacrylate resin (GMA). Although this would mean that the number of specimens would be fewer it afforded two advantages: the antigens of interest could be definitively identified as antibodies had been previously tested on GMA embedded human tissue in the Histochemistry Research Unit; and GMA embedding is less likely to cause antigen cross-linking and thus the level of antigen recognition is higher.[195]

Processing and Embedding Specimens in Glycol Methacrylate

Fresh pancreatic tissue was diced into small cubes of approximately 1mm^3 and then placed immediately into ice cold acetone (Fisher Scientific) containing the protease inhibitors iodoacetamide (20mM) (Sigma) and phenylmethanesulfonylfluoride (2mM) (Sigma). The tissue was then incubated in the fixative overnight at -20ºC, before processing the following day into water-permeable GMA as follows and as also described by Britten *et al*.[195] The fixative was replaced with fresh, high grade acetone at room temperature for 15 minutes and then methyl benzoate (BDH Laboratory Supplies) for a further 15 minutes. The pancreatic tissue was subsequently infiltrated with a processing solution composed of 5% methyl benzoate in glycol methacrylate solution A (Polysciences) (See Appendix) at 4°C, which was changed after 30, 90, 180 and 270 minutes. The biopsies were finally embedded within flat bottomed capsules (TAAB), in newly prepared GMA embedding solution. These were allowed to polymerise overnight at 4°C. Following polymerisation the GMA blocks were stored for the long term in airtight boxes containing anhydrous silica gel at -20°C.

Section Cutting

Following preliminary shaping of the GMA blocks using a file and razor blade, 2µm sections were cut using an Ultracut Rotary Microtome (Leica, Heidelberg) and floated

onto 0.2% ammonia (Fisher Scientific) solution in water for one minute and dried on poly-L-lysine (PLL) coated slides (Cellpath) at room temperature for 1 hour. Initially sections were cut for staining with toluidine blue (Merck) to assess tissue architecture and suitability for immunohistochemical analysis. Each pancreatic tissue sample was then cut in two series of 8 sections. Every slide also contained a sample of human tonsillar tissue as a positive control (See Figure 10).

Figure 10: Slide Layout Following Section Cutting

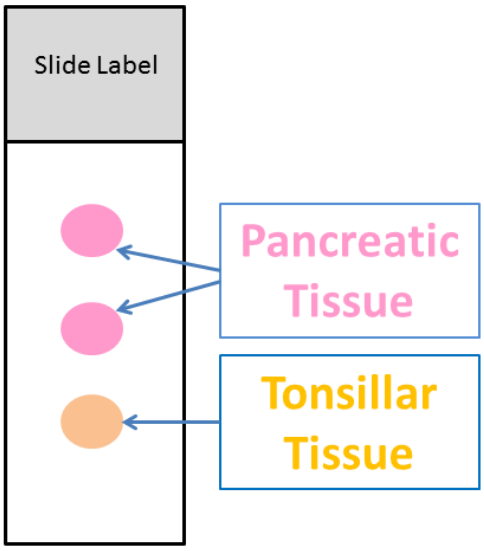


Figure 10 Legend

Pancreatic and tonsillar tissue was cut and dried onto poly-L-lysine coated slides. Each slide contained two sections of pancreatic tissue and one section of tonsillar tissue (to serve as a positive control) for each antibody and for every patient.

Streptavidin Biotin Peroxidase Indirect Staining Technique

Firstly endogenous peroxidase activity was blocked using a pre-treatment solution of 0.1% sodium azide (Merck) and 0.3% hydrogen peroxide (Sigma), in distilled water for 30 minutes. Following this, the slides were washed with tris buffered saline (TBS) (Sigma) and non-specific binding sites were blocked by addition of blocking medium (See Appendix) for 30 minutes. After draining, the sections were incubated overnight with the primary antibodies at their appropriate dilutions (See Table 7), at room temperature, overlaid with coverslips (Cellpath) to reduce evaporation.

After 18-24 hours, the coverslips were removed and the sections were washed with TBS, before addition of a rabbit anti-mouse biotinylated secondary antibody (Dako), at a concentration of 1:1,000, for 2 hours. The sections were then washed with TBS before adding avidin-biotin-complex reagents A + B (Vectastain), at a concentration of 1:75, for a further 2 hours. The sections were then: washed with TBS; stained with the chromagen acetone 3-amino-9-ethylcarbazole (AEC) (Genetex) for 20 minutes; washed in TBS and then running tap water; counterstained with Mayers haematoxylin

(See Appendix) for 1 minute; and then washed in running tap water again. Finally the slides were mounted by applying Crystalmount (Biomedia), which was baked to dryness in an oven at 80°C, prior to the addition of coverslips using Pertex mounting medium (Cellpath).

Table 7: Primary Antibodies Employed for GMA Immunohistochemistry

Antibody Target	Manufacturer	Clone	Optimal Dilution
CD3	AbD Serotec	UCHT1	1:700
CD4	Dako	4B12	1:25
CD8	Dako	DK25	1:100
FoxP3	Abcam	236A/E7	1:75
IFN-γ	R&D Systems	25718	1:10
IL-13	R&D Systems	32116	1:50
IL-17a	R&D Systems	41809	1:25

Controls

Positive and negative controls were included in every immunohistochemistry staining run. Negative control was achieved by omission of the primary antibody and its replacement with TBS. Human tonsil, which is rich in lymphocytes of all types, was used as a positive control.

Assessment and Quantitation

The area of pancreatic tissue was calculated for each section using a video computer image analysis system (Zeiss KS400 on an Axioskop 2 microscope). Nucleated cells showing positive red staining were counted in each section using a Leica light microscope at x400 magnification. I was blinded to the patient group at all times. Results were expressed as the number of positive cells per mm^2 of pancreatic tissue.

Histological Examination of GMA Embedded Pancreatic Tissue

All pancreatic tissue analysed had dual histological confirmation of either chronic pancreatitis or normality: A haematoxylin stained negative control slide of each patient was reviewed by an independent consultant histopathologist who was blinded to the

specimens origin; and a histopathology department report was issued for the clinical non-research tissue. The independent review correlated with the histopathology department report in all cases.

STATISTICAL ANALYSIS

Advice was sought from Dr. Scott Harris at the University of Southampton's Department for Medical Statistics. Following thorough discussion and review it was clear that as the data was skewed and frequently contained fewer than 30 samples, non-parametric tests should be performed e.g. Mann-Whitney U and Wilcoxon Signed Rank Tests. Data was analysed using PASW Statistics 18.0.0 (formerly SPSS).

Chapter 3: Development and Validation of Methods

DEVELOPMENT OF A TECHNIQUE TO ISOLATE LYMPHOCYTES FROM PANCREATIC TISSUE

Surgical resection of pancreatic tissue is most commonly undertaken to treat malignant disease, in particular pancreatic adenocarcinoma. Patients with chronic pancreatitis undergo surgery infrequently thus tissue is scarce. Even in a regional pancreatic surgical centre such as Southampton General Hospital only twenty pancreatic resections are undertaken each year for benign disease; most of which are for chronic pancreatitis.[196] Isolation of lymphocytes from tissue allows their study in great detail and therefore developing a robust and reproducible technique to isolate them from chronically inflamed and normal pancreatic tissue was central to establishing the research project.

Normal pancreatic tissue contains few lymphocytes however during the development of chronic inflammatory and malignant pancreatic disease, lymphocytes infiltrate the organ in significant numbers. Previously, analysis of pancreas infiltrating lymphocytes has most frequently been undertaken using immunohistochemistry. Only two groups, one at the University of Bern in Switzerland [133, 197] and one at the University Hospital of Heidelberg in Germany,[139] have isolated lymphocytes from human chronic pancreatitis tissue, using two differing protocols. The latter group's most recent publication (Schmitz-Winnenthal *et al* 2010)[139] post-dated the initiation of this development process. Therefore a standard, well-established and widely reproduced protocol for isolating lymphocytes from chronic pancreatitis tissue was not available. However several groups have developed methods for isolating lymphocytes from pancreatic tissue of non-obese diabetic (NOD) mice; and a few have developed techniques for isolating lymphocytes from human and mouse pancreatic adenocarcinoma (PAC) tissue (See Table 8). I therefore devised a protocol based on the published works of others that describe lymphocyte isolation from pancreas and bowel, and augmented them with new reagents and techniques. Direct comparison of the techniques allowed me to rationalise the best protocol. These methods are described in detail in Chapter 2: Methods - Isolating Lymphocytes from Pancreatic Tissue.

The isolation of lymphocytes from pancreatic tissue requires two main steps:

1) Digestion of tissue to disaggregate cells and create a single cell suspension
2) Separation of lymphocytes from the cellular suspension

These two processes and the development of the protocol will now be discussed in more detail.

Table 8: Previous Digestion Methods Used for Isolating Pancreatic Lymphocytes

Author	Animal / Disease	Mechanical Method	Enzyme
Garza *et al* [198]	NOD Mouse	Mince with Scissors Cell Strainer / Mesh	Nil
Hawkins *et al* [199] Trembleau *et al* [200]	NOD Mouse	Mince with Scissors	Collagenase IV
Kendall *et al* [201]	NOD Mouse	Mince with Scissors	Collagenase P
Lenschow *et al* [202]	NOD Mouse	Mince with Scissors Cell Strainer / Mesh	Collagenase P DNase
Gnerlich *et al* [203]	Mouse PAC	Mince with Scissors gentleMACS Dissociator	Collagenase DNase Hyaluronidase
Liyanage *et al* [84]	Human PAC	Mince with Scissors	Collagenase (unspecified)
Hunger *et al* [133] Saurer *et al* [197]	Human CP	Mince with Scissors Cell Strainer / Mesh	Collagenase V
Schmitz-Winnenthal *et al* [139]	Human CP	Mince with Scissors Cell Strainer / Mesh	Nil

Digestion of Pancreatic Tissue and Creation of a Single Cell Suspension

The disaggregation of tissue may be performed using either mechanical and/or chemical/enzymatic means. Whilst the majority of methods use both techniques some have favoured using mechanical digestion alone (See Table 8). However each of the

techniques employed on animal or human pancreatic tissue has potential problems: Tissue may not be adequately digested preventing lymphocyte isolation; or tissue may be over-digested causing destruction of cell surface antigens or cell death.

Methods Used in Mice

The methods used to digest the pancreatic tissue of NOD mice (See Table 8) are not fully applicable to the digestion of human CP tissue as the former does not have significant fibrosis. It is therefore likely that using any of these methods would result in failure to appropriately digest the pancreatic tissue and liberate lymphocytes for subsequent isolation. However the methods used to digest mouse PAC tissue are more applicable to human CP tissue as PAC in mice and humans is associated with an intense desmoplastic reaction with associated tissue fibrosis.[204]

Mechanical Digestion

As human chronic pancreatitis tissue may be significantly fibrotic it may not be sufficiently digested by mechanical means alone (See Table 8). However to provide a good surface area for proteolytic enzymes to work on it is beneficial to mechanically digest it first. Some groups had used a gentleMACS Dissociator, an instrument for the automated dissociation of tissues into single cell suspensions or homogenates. However I decided that mincing with scissors seemed to be the most reasonable and economically viable means to mechanically digest tissue as it did not require the purchase of new, expensive equipment or potentially result in homogenisation of the tissue preventing analysis of single cells.

Enzymatic Digestion

Digestion of pancreatic tissue using chemical means, most commonly using collagenase, is widely accepted for the isolation of islets and pancreatic stellate cells from human and rat pancreata.[35, 36, 205-207] Collagenase has also been used to digest pancreatic tissue from mice in the process of isolating lymphocytes (See Table 8).[199-202] The mice however did not have significant pancreatic fibrosis. Hunger *et al*, in a seminal paper, were the first to describe a technique of digesting human chronic pancreatitis tissue using mechanical digestion with scissors followed by enzymatic digestion with Collagenase V.[133]

Several collagenases exist in a number different formulations and strengths. Following personal communication with Dr Sylvia Pender, a Senior Lecturer at the University of Southampton, it was suggested that Collagenase II would be the most appropriate to

digest human chronic pancreatitis tissue whilst maintaining lymphocyte antigenicity. Her experience had shown that when digesting fibrotic gastrointestinal tissues Collagenase IV, V and P destroyed lymphocyte cell membrane antigens resulting in reduced ability to identify them using flow cytometry. Furthermore the latter are only recommended for the isolation of hepatocytes, islets of Langerhans and pancreatic stellate cells by the manufacturers. Collagenase II was therefore chosen. Additionally a fine balance exists between exogenous tissue digestion using collagenase and endogenous tissue digestion related to the release of pancreatic enzymes from tissue. I therefore initially used Collagenase II at a concentration of 1mg/ml, but also analysed whether a higher concentration of 2mg/ml produced higher lymphocyte yields, which it did not.

To produce a single cell suspension from digested tissue a widely employed technique is to push it through a mesh. Following initial mechanical digestion with scissors and subsequent enzymatic digestion with Collagenase II (at 37°C for 30 minutes) the sample was passed through a 100µm Falcon cell strainer. I also opted to use deoxyribonuclease (DNase) to prevent cell agglutination; an approach previously used by Lenschow, Gnerlich and Apte.[35, 202, 203]

Solutions for Washing Cells and Maintaining Viability

To maintain cell viability and remove proteolytic enzymes the cells would need to be washed a number of times. I therefore decided upon the following:

- Samples should be kept ice cold, unless being actively enzymatically digested in an incubator, to reduce unwanted enzyme activity;
- Hanks balanced salt solution (HBSS) would be used to make up Complete Medium (See Appendix) as it has a higher buffering capacity than PBS;
- As collagenase is calcium dependent HBSS with calcium (HBSS+) should be used to make up any solutions containing it;
- Foetal calf serum (FCS) would be added to Complete Medium as it contains higher levels of growth factors and lower levels of antibodies than horse serum (as used by Bachem *et al* [133]).

The creation of a Lymphocyte Isolation Buffer (See Appendix) represented the most novel aspect of the protocol and was conceived in an attempt to protect cells from unwanted enzymatic degradation and damage by free radicals, and provide an environment which would preserve cells, provide essential substrates and maintain viability. The buffer was comprised of: PBS without calcium & magnesium, FCS (10%),

EDTA (1mM), N-acetyl cysteine (100μM), ascorbic acid (100μM), Pefabloc (400μM) and D-glucose (4.5 g/L). Serine proteases are dependent on the divalent cations calcium and magnesium. Therefore to inhibit intrinsic and extrinsic serine proteases following enzymatic digestion, PBS without calcium and magnesium was used, in addition to EDTA and N-acetyl cysteine (NAC) that chelate metal cations. NAC would also serve as an anti-oxidant, as well as ascorbic acid; and FCS and D-glucose would provide growth factors and a source of energy for cellular metabolism respectively.

I also added Pefabloc to the buffer, a compound used in the isolation of human pancreatic islets for transplantation. Pancreata removed from brain-dead heart beating donors are digested using liberase, a mixture of collagenase classes and thermolysin, in the presence of Pefabloc to inhibit endogenous protease activity. This method has been reproducibly shown to improve islet cell yield and viability.[205-207] As tissue from patients with chronic pancreatitis contains an extra-cellular matrix rich in collagen, I used the Lymphocyte Isolation Buffer after enzymatic digestion. This allowed endogenous proteases to aid the digestion process and then all enzymatic activity to be halted when desired.

Separation of Lymphocytes from the Cellular Suspension

Lymphocytes were purified from the resulting tissue cell suspension using density gradient centrifugation. This approach is in widespread use for separating less dense portions of a solution, mixture, or suspension from more dense portions by centrifugal force. I decided to use Percoll for this purpose as although there is little difference between Ficoll-Paque Plus and Percoll (Sigma), Percoll was used in the previously described techniques.[133, 197] The layering method was altered between the first two protocols in light of advice that, if in the future I wished to analyse other cell types in the suspension e.g. fibroblasts, it would be prudent to use a five layer technique (as per Percoll product information: #18-1115-69) (See Figure 7). Following density gradient centrifugation lymphocytes were washed in RPMI Wash Solution (See Appendix) which would maintain cell integrity better than Complete Medium or HBSS(+). Lymphocytes were then counted using a Leica light microscope and haemocytometer. Dead cells were excluded by uptake of trypan blue.

Results

Protocol 1 aimed to validate whether mechanical digestion or mechanical plus enzymatic digestion gave superior yields of lymphocytes: Method A employed mechanical digestion with scissors and a cell strainer; and Method B used mechanical

digestion with scissors followed by enzymatic digestion with Collagenase II (1mg/ml) then further cell dissociation with a cell strainer. Lymphocytes were counted using a haemocytometer and viability determined by trypan blue exclusion. The results of these initial experiments are shown in Table 9. At first glance it appeared that there was little difference between the two methods, however further analysis of the third tissue specimen using flow cytometry (starred result in Table 9) demonstrated that the visual count of lymphocytes in this instance was incorrect for Method A and significantly over-counted the numbers present. The true result appeared to be much closer to that for Method B. I therefore concluded that Method B was superior providing lymphocyte yields that were 7% - 19% higher.

Table 9: Results of Pancreatic Tissue Lymphocyte Isolation Using Protocol 1

Method / Tissue	Lymphocyte Cell Count (per mg pancreatic tissue)		
	PAC	CP (P04)†	CP (P09)†
Method A Pellet	317.5	13,580.2	6,978.2
Method A Supernatant	2,438.1	87,654.3	30,339.8
Method B Pellet	1,000.0	2314.8	726.7
Method B Supernatant	1,956.5	122,685.2	9,447.7
Method A Total	2,755.6	101,234.5	37,318.0*
Method B Total	2,956.5	125,000.0	10,174.4

Table 9 Legend

*Method A used mechanical digestion alone. Method B used both mechanical and enzymatic digestion (Collagenase 1mg/ml). Lymphocytes were counted using a haemocytometer and viability determined by trypan blue exclusion. The pancreatic tissue of one patient with pancreatic adenocarcinoma (PAC) and two patients with chronic pancreatitis (CP) was assessed. † Each patient is identified in the demographic data shown in Table 13. * Spurious result; it was similar to that for Method B.*

Protocol 2 was then devised to establish the optimal concentration of collagenase II. I initially began with comparing 1mg/ml and 2mg/ml. The results are shown in Table 10.

Table 10: Results of Pancreatic Tissue Lymphocyte Isolation Using Protocol 2

Method / Tissue	Lymphocyte Cell Count (per mg pancreatic tissue)		
	CP (P13)†	CP (P19)†	N (P22)†
Collagenase 1 mg/ml - Pellet	3,714.3	46.1	0
Collagenase 1 mg/ml - Supernatant	148,571.1	322.3	0
Collagenase 1 mg/ml - Total	152,285.4	368.4	0
Collagenase 2 mg/ml - Pellet	9,750.0	0	0
Collagenase 2 mg/ml - Supernatant	37,500.0	0	0
Collagenase 2 mg/ml - Total	47,250.0	0	0

Table 10 Legend

These experiments were initially used to establish the optimal concentration of Collagenase II for enzymatic digestion: 1mg/ml or 2 mg/ml. The pancreatic tissue of two patients with chronic pancreatitis (CP) and one patient without pancreatic disease (normal, N) and was assessed. Lymphocytes were counted using a haemocytometer and viability determined by trypan blue exclusion. As the pellet obtained always contained far fewer lymphocytes than the supernatant (also See Table 9) Protocol 3 was devised, however its main purpose was still to evaluate optimal collagenase concentration. † Each patient is identified in the demographic data shown in Table 13 and Table 14.

Protocol 2 was then improved to cut down the complexity of the lymphocyte isolation technique. It became clear that the initial pellet obtained following centrifugation (400rpm for 3 minutes at 4ºC) contained a small fraction of the total number of lymphocytes compared to the supernatant. This initial centrifugation step was designed to remove cellular debris and not lymphocytes, however this was clearly not working. A longer and faster centrifugation step (1500rpm for 5 minutes at 4°C) was therefore employed to collect all cells, as is routinely performed for the separation of PBMC in a solution. The results of protocol 3, which was still primarily designed to compare the effect of collagenase concentration on lymphocyte yields, is shown in Table 11. Further increments in the concentration of collagenase employed were not required as the results showed little difference existed between the use of 1mg/ml or 2mg/ml.

Table 11: Results of Pancreatic Tissue Lymphocyte Isolation Using Protocol 3

	Lymphocyte Cell Count (per mg pancreatic tissue)				
Tissue	CP (P23)†	N (P26)†	CP (P29)†	N (P33)†	CP (P34)†
Collagenase 1 mg/ml	37,276.0	392.9	1,769.9	101.6	23,793.3
Collagenase 2 mg/ml	39,453.1	604.8	0	125.9	10,699.0

Table 11 Legend

These experiments aimed to establish the optimal concentration of Collagenase II used for enzymatic digestion: 1mg/ml or 2 mg/ml. Lymphocytes were counted using a haemocytometer and viability determined by trypan blue exclusion. The pancreatic tissue of three patients with chronic pancreatitis (CP) and two patients without pancreatic disease (normal, N) was assessed. † Each patient is identified in the demographic data shown in Table 13 and Table 14.

Conclusion

The optimal method for liberating live lymphocytes from pancreatic tissue required the use of both mechanical and enzymatic digestion. The creation of a Lymphocyte Isolation Buffer, although it was not compared with other solutions, is likely to have contributed to improved lymphocyte survival, and the use of Collagenase II at a low concentration of 1mg/ml preserved lymphocyte surface antigenicity well. The final protocol and definitive technique used for isolating lymphocytes from pancreatic tissue proved to be highly reproducible and was central to the success of the research.

COMPARING FLOW CYTOMETRY ANALYSIS OF FRESH AND CRYOPRESERVED LYMPHOCYTES

Principles

I planned to undertake a number of different experiments on each patient's donated samples. As it was not technically feasible to perform all of the experiments immediately subsequent to isolation of PBMC or mononuclear cells from pancreatic tissue I elected to cryopreserve harvested cells. A number of papers have evaluated the effect of cryopreservation on cell viability and function as assessed by flow cytometry. They conclude that cryopreservation has no effect on the measurable number of CD3+CD4+ or CD3+CD8+ T-cell subsets and that cryopreserved PBMC have consistent proliferative responses and are suitable for functional analyses i.e. cytokine detection.[208-210] Some authors however have reported a reduction in CD3+CD4+ T-cells and Treg cell numbers in cryopreserved samples. Nonetheless there was a strong correlation between Treg cell frequency in fresh and frozen PBMC suggesting that determination in either fresh or frozen samples was appropriate but not a mixture of both samples.[211, 212]

I therefore aimed to establish how cryopreservation can affect the lymphocyte populations of interest by conducting a short experimental protocol. This contained few samples and therefore was not powered to establish statistical significance. It would however allow me to develop a better understanding of how cryopreservation could alter specific lymphocyte subsets. I would then be able to determine, in light of previous work by others, how to create a legitimate and reproducible study protocol.

Method

Peripheral venous blood was taken from three healthy individuals (See Table 14: P53, P54, P56). PBMC were then isolated and split into two fractions: One was cryopreserved and the other was analysed immediately using flow cytometry. The cryopreserved samples were thawed within one week and examined in an identical manner using flow cytometry. All four panels of antibodies were employed. All methods used were performed as previously described. It should be noted that it is not possible to statistically analyse this paired parametric data, using e.g. the Wilcoxon Signed Ranks Test, as the number of samples is less than seven. However visual assessment of the means and medians provides a reasonable way of detecting any obvious differences.

Results

The mean and median results of all lymphocyte subsets analysed are shown in Table 12. Cryopreservation has little or no discernible effect on CD3+, double negative, CD4+ and CD8+ T-cells (including memory / naïve subsets and α4β7 integrin or CD161 expressing cells) as shown by inspection of the separation of mean and median values of fresh and cryopreserved samples. A reduction in the proportion of activated $CD4^+CD25^+$ T-cells is evident however (mean/median expressed as % of $CD4^+$: 25.97 *vs.* 20.97 / 26.05 *vs.* 21.12). There is also a reduction in the proportion of $CD4^+CD25^+FoxP3^-$ cells (mean/median expressed as % of $CD4^+$: 20.50 *vs.* 13.23 / 20.50 *vs.* 14.30) with a corresponding rise in $CD4^+CD25^+FoxP3^+$ Treg cells (mean/median expressed as % of $CD4^+$: 5.47 *vs.* 7.74 / 5.55 *vs.* 6.82).

T-helper cell analysis was flawed in these experiments. The serial analysis of fresh and frozen T-helper cells was therefore abandoned until the technique was perfected. At this point it was decided that as all of the T-cell analysis was going to be performed on cryopreserved cells it would not be repeated for these purposes alone. I believe that my initial failures in stimulating and staining T-helper cell subsets was related to three significant components:

1. I diluted, aliquoted and froze the ionomycin. This alters the protein's confirmation rendering it ineffective at stimulating T-cells;
2. I was using an old stock of Permeabilisation buffer. Ageing Permeabilisation buffers lose efficacy as the active moiety Saponin forms micro-crystals within the solution;
3. I was over-cautious in vortexing cells following permeabilisation. This prevented adequate concentrations of antibody entering the lymphocytes.

The analysis of CD8+ and double negative (DN) mucosal-associated invariant T (MAIT) cells before and after cryopreservation cannot also be fully interpreted as the spread of the data is large, as shown by significant differences in mean and medians, and the effect of freezing variable (See Figure 11). In two cases cryopreservation decreased the proportion of both CD8+ and DN MAIT (as a percentage of their parent group), but in one case it increased the detectable proportion.

Table 12: Fresh and Cryopreserved Lymphocyte Analysis Using Flow Cytometry

Lymphocyte Subset	Fresh		Cryopreserved	
	Mean	Median	Mean	Median
CD3+ (% of lymphocytes)	66.73	73.70	68.87	68.10
CD4+ (% of CD3+)	59.23	59.50	61.97	62.30
CD4+ Memory (% of CD4+)	57.00	60.90	54.83	57.00
CD4+ Naïve (% of CD4+)	43.00	39.10	45.17	43.00
CD4+ Integrin α4β7+ (% of CD4+)	9.38	11.30	9.30	8.74
CD8+ (% of CD3+)	31.37	30.60	28.87	28.10
CD8+ Memory (% of CD8+)	50.57	54.70	48.90	49.90
CD8+ Naïve (% of CD8+)	49.43	45.30	51.10	50.10
CD8+ Integrin α4β7+ (% of CD8+)	18.17	18.60	19.43	18.90
CD8+ CD161+ (% of CD8+)	8.91	7.94	7.66	5.90
CD8+ MAIT (% of CD8+)	9.49	2.65	9.95	12.80
DN (% of CD3+)	6.16	6.08	5.44	5.68
DN MAIT (% of DN)	13.11	8.71	17.26	21.30
Th1 (% of CD4+)	0.86	0.45	0.58	0.36
Th2 (% of CD4+)	1.48	0.61	0.61	0.64
Th17 (% of CD4+)	1.13	0.70	0.63	0.53
Treg (% of CD4+)	5.47	5.55	7.74	6.82
CD4+ CD25+ FoxP3- (% of CD4+)	20.50	20.50	13.23	14.30
CD4+ CD25+ (% of CD4+)	25.97	26.05	20.97	21.12

Table 12 Legend

PBMC were isolated from three healthy controls. Paired analysis of fresh and cryopreserved lymphocytes was performed using flow cytometry. DN – Double negative; MAIT – Mucosal-associated invariant T-cell ($CD3^+$ $CD4^-CD8^+/CD4^-CD8^-$ $CD161^+$ TCR $V\alpha7.2^+$); Th – T-helper; Th1 – $CD4^+$ IFN-γ^+; Th2 – $CD4^+$ IL-13^+; Th17 – $CD4^+$ IL-17^+; Treg – T-regulatory cell ($CD4^+$ $CD25^+$ $FoxP3^+$ $CD127^{-/lo}$).

Figure 11: Paired Dot Plots Comparing Fresh and Cryopreserved MAIT Cells

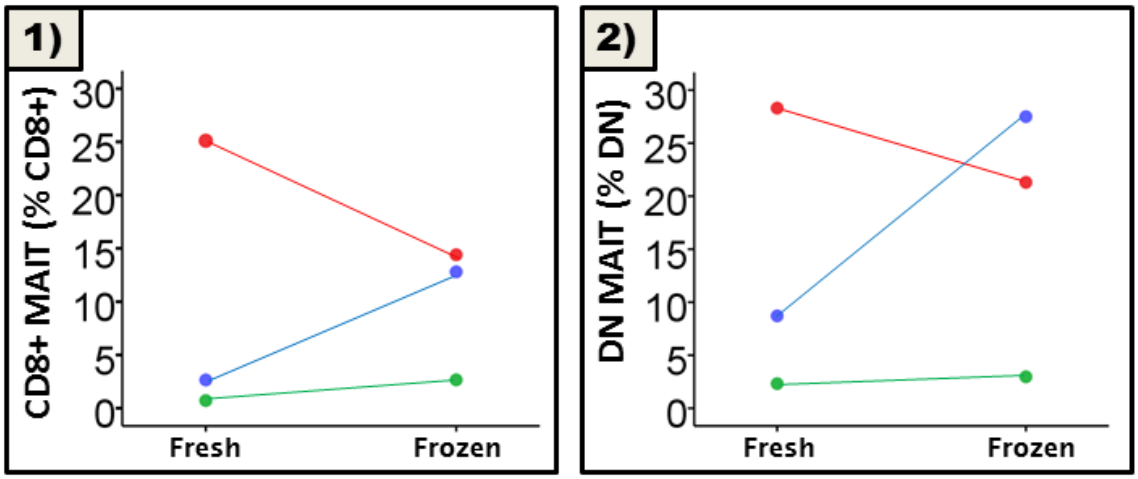


Figure 11 Legend

Three healthy individuals' PBMC were analysed with flow cytometry before and after cryopreservation. The results for CD8+ (1) and double negative (DN) MAIT cells (2) are shown. The colours of each dot and line represent an individual patient.

Conclusion

It is tempting to speculate on a number of the findings despite the shortcomings of the study, in particular its small sample size. It appears that cryopreservation had little or no effect on the majority of CD3+, CD4+ CD8+ and DN T-cells, which is consistent with previous studies.[209, 212] Cryopreservation did however reduce the proportion of activated $CD4^+CD25^+$ T-cells. This may be related to the larger size of activated T-cells and their increased metabolic activity, which exposes a greater surface area to the cold thus enhancing their susceptibility to cellular stress. The reason that $CD4^+CD25^+FoxP3^+$ Treg cells increase in number following cryopreservation is more difficult to explain. It may merely be a manifestation of a change in the linked proportions of $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^+FoxP3^-$ T-cells; as there is a reduction in the proportion of $CD4^+CD25^+FoxP3^-$ T-cells there may be a corresponding rise in the proportion of $CD4^+CD25^+FoxP3^+$ Treg cells. Alternatively it could be that as Treg cells function to dampen inflammatory responses their survival is complicit with living in an "hostile" environment in which injurious stimuli are frequent but have little effect in their viability and vitality; this provides them with a survival advantage when faced with the cellular stresses imposed by cryopreservation. It should be noted that this result is at odds with the work of previous authors. Elkord *et al* found that the proportion of Treg cells was reduced in all PBMC samples that were analysed after cryopreservation.[211] Their study contained more samples (n=6) and even though this is

still small in experimental terms there was uniformity between all samples analysed suggesting a true result.

Overall the analysis highlights the importance of maintaining a uniform protocol for analysing mononuclear cells. Furthermore analysis of a significantly larger dataset is required to discover if cryopreservation truly alters the proportion of specific lymphocyte populations as the number of samples analysed in published studies is often small.

Chapter 4: Results

In the introduction I reviewed the literature and described the accumulating evidence that supports the role of lymphocytes in the pathogenesis of chronic pancreatitis. The specific components of the pancreatic tissue lymphocytic infiltrate and aberrations in the composition of peripheral blood lymphocytes are poorly described and the literature inconclusive. This may be related to the fact that chronically inflamed pancreatic tissue is not readily available and techniques to isolate lymphocytes from it are not well described. Once I had generated a reproducible method to extract mononuclear cells from pancreatic tissue further analysis was possible. This aimed to test the following hypotheses:

1) The peripheral blood of patients with chronic pancreatitis is comprised of different proportions of T-cell subsets
2) Specific T-lymphocyte subsets are enriched in chronic pancreatitis tissue
3) The proportion of T-regulatory cells to T-helper cells, in particular IFN-γ and IL-17 secreting cells, is skewed in the pancreatic tissue and peripheral blood of patients with chronic pancreatitis
4) Mucosal-associated invariant T-cells infiltrate chronically inflamed pancreatic tissue and are accordingly depleted in peripheral blood

The presence of pro-inflammatory T-lymphocytes in chronic pancreatitis tissue and the potential dysregulation of T-lymphocyte function and homoeostasis in both pancreatic tissue and peripheral blood is likely to perpetuate damage triggered by acinar cell injury. Establishing the precise nature of the lymphocytic infiltrate in CP is therefore essential if this is to be further investigated. I therefore endeavoured to perform a phenotypic analysis of intrapancreatic and peripheral blood lymphocytes in patients with CP using immunohistochemistry and flow cytometry to identify T-lymphocytes. Furthermore I sought to functionally characterise CD4+ T-helper cells (Th1/Th2/Th17) and T-regulatory cells in pancreatic tissue and blood of patients with chronic pancreatitis using flow cytometry.

PATIENT DEMOGRAPHICS

Peripheral blood and pancreatic tissue was collected from twenty nine patients with chronic pancreatitis and twenty controls (See Table 13 and Table 14).

Table 13: Demographics of Chronic Pancreatitis Patients

Identifier	Age	Gender	Aetiology					Signs / Symptoms		Pancreatic Resection (Indication)
			Alcohol (Still Drinking)	Smoker	Hypercalcaemia	Pancreatic Duct Obstruction	Idiopathic	Exocrine Insufficiency	Pain	
P02	39	M	Y (n)	Y	N	N	N	N	Y	N
P04	35	M	Y (n)	Y	N	N	N	N	Y	Y (pain)
P08	47	M	Y (n)	Y	N	N	N	Y	Y	N
P09	44	M	Y (n)	N	N	N	N	N	Y	Y (pain)
P11	74	M	Y (y)	Y	N	N	N	Y	Y	N
P13	39	M	Y (n)	Y	N	N	N	Y	Y	Y (pain)
P14	41	M	Y (y)	Y	N	N	N	Y	Y	N
P15	51	M	Y (n)	Y	N	N	N	Y	Y	Y (pain)
P16	47	M	N	N	N	N	Y	Y	Y	N
P17	39	M	Y (y)	Y	N	N	N	N	Y	N
P18	61	M	N	N	N	N	Y	Y	N	N
P19	46	M	Y (n)	Y	N	N	N	Y	Y	Y (pseudocyst)
P20	67	M	Y (n)	Y	N	N	N	Y	N	N
P21	66	M	Y (n)	N	N	N	N	N	Y	N
P23	47	M	N	N	N	Y	Y	Y	Y	Y (pain)
P24	25	M	Y (n)	Y	N	N	N	Y	Y	N
P25	32	M	Y (y)	N	N	N	N	Y	Y	N
P28	51	M	Y (n)	Y	N	N	N	Y	Y	N
P29	62	M	Y (n)	Y	N	Y	N	N	Y	Y (pain)
P30	45	M	Y (y)	Y	N	N	N	N	Y	N
P32	42	M	Y (y)	Y	N	N	N	Y	Y	N
P34	43	M	Y (y)	Y	N	N	N	N	Y	Y (mass)
P35	32	M	Y (y)	Y	N	N	N	Y	Y	N
P36	64	F	Y (n)	Y	N	N	N	N	Y	N
P37	50	M	Y (n)	Y	N	N	N	Y	Y	N
P39	58	M	Y (n)	Y	N	N	N	Y	N	N
P42	61	F	N	Y	N	N	Y	Y	Y	N
P44	63	M	Y (n)	Y	N	Y	N	N	Y	Y (pain)
P46	62	M	N	Y	Y	N	N	Y	Y	Y (pain)

Table 14: Demographics of Control Patients

Identifier	Age	Gender	Diagnosis	Excess Alcohol	Smoker
P07	75	F	Pancreatic Mucinous Cystic Neoplasm	N	N
P10	64	M	Neuroendocrine Tumour	N	N
P12	47	F	Pancreatic Mucinous Cystic Neoplasm	N	N
P22	45	M	Ampullary Cancer	Y	N
P26	68	M	Cholangiocarcinoma	N	Y
P33	75	F	Pancreatic Neuroendocrine Tumour	N	N
P40	73	M	Ampullary Cancer	N	N
P41	48	M	Cholecystectomy Follow-Up	N	Y
P43	67	M	Pre-Operative Inguinal Hernia	N	N
P45	62	F	Cholangiocarcinoma	N	N
P47	61	M	Benign Prostatic Hypertrophy	N	N
P48	52	M	Ureteric Stone Follow-Up	N	N
P49	60	M	Ureteric Stone Follow-Up	N	N
P50	42	F	Ureteric Stone Follow-Up	N	N
P51	47	M	Ureteric Stone Follow-Up	N	N
P52	39	M	Ureteric Stone Follow-Up	N	Y
P53	35	M	Healthy	N	Y
P54	34	M	Healthy	N	N
P55	37	M	Healthy	N	N
P56	32	M	Healthy	N	N

T-LYMPHOCYTE SUBSETS IN PERIPHERAL BLOOD

Cryopreserved PBMC isolated from chronic pancreatitis patients and controls were analysed using flow cytometry as previously described. The blood of 26 patients with CP and 16 controls was assessed (See Table 15). Live T-cell subsets were identified as being $CD3^+$, and either $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ (double negative, DN) (See Figure 12). T-cytotoxic cells were demonstrated by positive staining for CD8 and CD161, and memory/naïve T-cells were shown by the presence or absence of CD45RO (See Figure 13). Finally gut homing T-cells were characterised by positive staining for integrins α4 and β7 (See Figure 14). Statistical analysis was undertaken using PASW Statistics 18.0.0 (formerly SPSS) employing a Mann-Whitney U Test.

No difference in the proportion of $CD3^+$ T-cells was found in the peripheral blood of chronic pancreatitis patients compared to controls (80.45% *vs.* 74.5%; p=0.282). However a trend towards a lower proportion of $CD4^+$ T-cells was noted (CP 66.65% *vs.* 73.10% control; p=0.076), which also led to a trend for an altered CD4:CD8 ratio (CP 2.80 *vs.* 3.53 control; p=0.097). Interestingly no statistical difference existed in the proportion of CD8+ (CP 23.95% *vs.* 20.85% control; p=0.130) or DN T-cells (CP 4.96% *vs.* 4.30% control; p=0.288), however the results do suggest the possibility of a higher proportion of CD8+ T-cells being present in the blood of CP patients (See Table 16 and Figure 15). The flow cytometry plots for every patients' CD3+ T-cells, showing CD4+, CD8+ and DN T-cells can also be seen in Figure 39 in the Appendix.

Further analysis of CD4+ and CD8+ T-cells revealed that no difference in the proportion of memory to naïve cells existed (See Table 16). Additionally no statistical difference was observed in the proportion of $CD8^+CD161^+$ cytotoxic T-cells (CP 5.80% *vs.* 9.08% control; p=0.126), however there was a suggestion that a difference might exist if a larger number of samples was analysed as the medians for each data set are quite different but the spread of data too large to produce statistically significant results (See Figure 15).

Identification of integrin α4β7+ cells demonstrated a trend towards a lower proportion of $CD8^+α4β7^+$ T-cells in the blood of CP patients compared to controls (CP 3.90% *vs.* 7.73% control; p=0.052), which was not evident in $CD4^+α4β7^+$ T-cells (CP 2.31 *vs.* 3.39 control; p=0.214) (See Figure 15). The flow cytometry plots for all patients' $CD8^+α4β7^+$ T-cells is shown in Figure 40 in the Appendix.

Table 15: Patient Demographics of Peripheral Blood T-Lymphocyte Subsets Analysed using Flow Cytometry

	Chronic Pancreatitis		Control	
Total Number	26		16	
Gender	24 Male / 2 Female		11 Male / 5 Female	
Median Age (years)	48.50	47.0 M 62.5 F	56.0	52.0 M 62.0 F
Age Range (years)	25 – 74	25 – 74 M 61 – 64 F	32 - 75	32 – 73 M 42 – 75 F

Table 15 Legend

The specific demographic details for each chronic pancreatitis patient (P02, P04, P08, P09, P11, P13, P14, P15, P16, P18, P19, P20, P21, P23, P24, P25, P28, P29, P34, P35, P36, P37, P39, P42, P44, P46) and control patient analysed (P07, P12, P22, P26, P33, P40, P43, P45, P47, P48, P49, P50, P51, P52, P53, P56) can be seen in Table 13 and Table 14 respectively.

Figure 12: PBMC Analysis Using Flow Cytometry Demonstrating the Basic Gating Used to Identify T-Cells

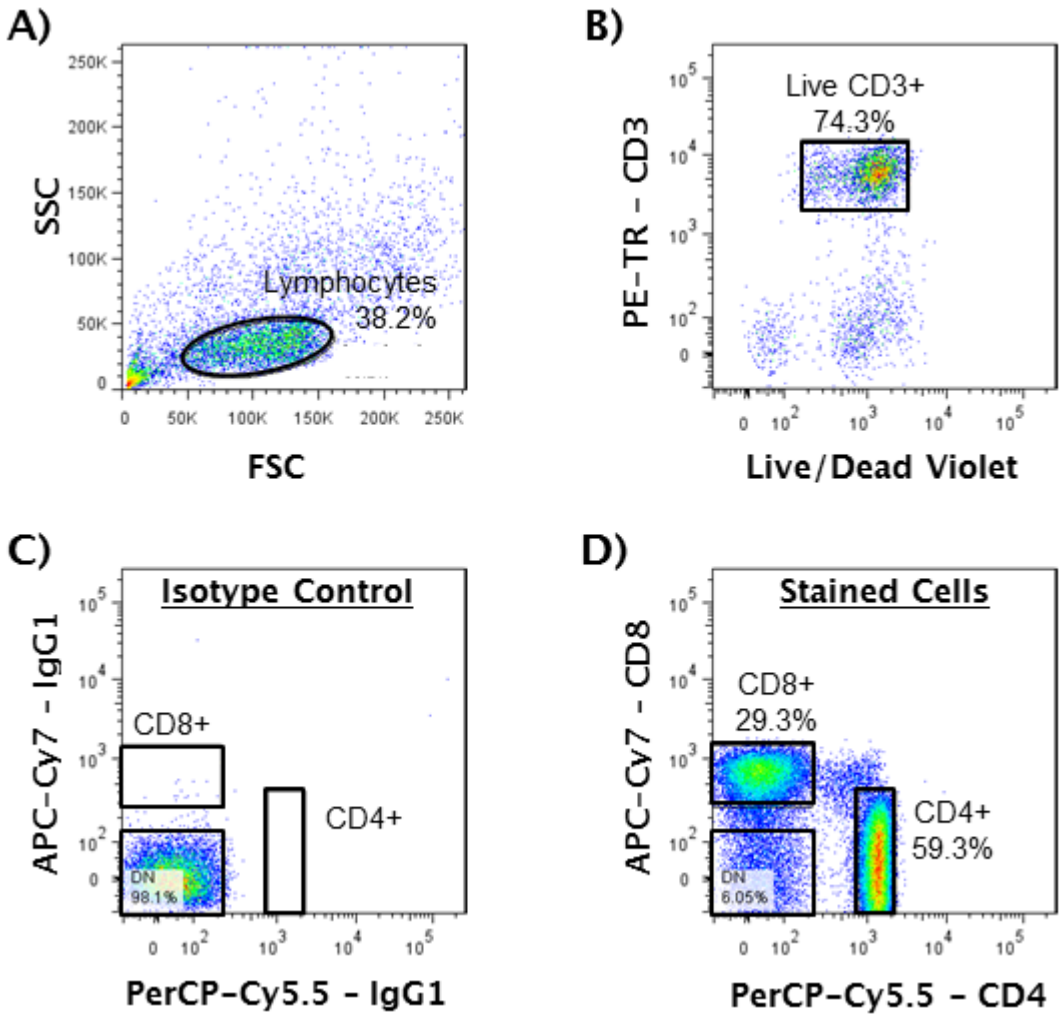


Figure 12 Legend

Peripheral blood mononuclear cells isolated from the blood of a healthy donor were stained with fluorochrome conjugated antibodies and dyes allowing identification of T-cell subsets. A representative sample is shown. A) Lymphocytes are characterised by specific forward scatter (FSC) and side scatter (SSC) properties allowing a lymphocyte gate to be created. B) Live CD3+ T-cells may then be identified within the lymphocyte gate. The live-dead violet dye adheres to cellular amines, thus a significant shift to $>10^4$ indicates cellular death. C) Live CD3+ T-cells represent the next step in the gating hierarchy. Isotype control antibodies confirm the correct position of the gates set in D) in which CD4+, CD8+ and double negative (DN) T-cells are identified.

Figure 13: Flow Cytometry Analysis of PBMC Identifying CD161+ and CD45RO+ T-Cells

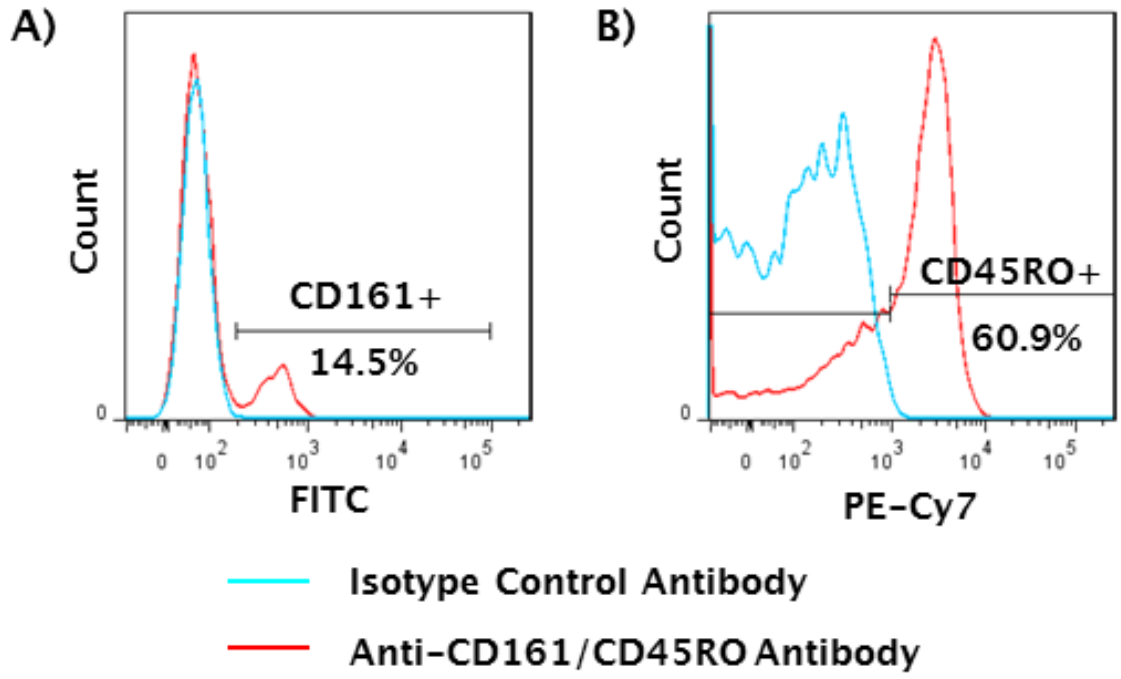


Figure 13 Legend

PBMC were isolated from a healthy blood donor. A) CD161 overlay histogram showing establishment of a positive gate using FITC labelled anti-CD161 and FITC labelled mouse IgG1 isotype control antibody (gated on CD8+ T-cells). B) CD45RO overlay histogram showing establishment of a positive gate using PE-Cy7 labelled anti-CD45RO and PE-Cy7 labelled mouse IgG2a isotype control antibody (gated on CD4+ T-cells). This was also performed for CD8+ T-cells (not shown).

Figure 14: Flow Cytometry Analysis of PBMC Identifying Integrin A4B7+ T-Cells

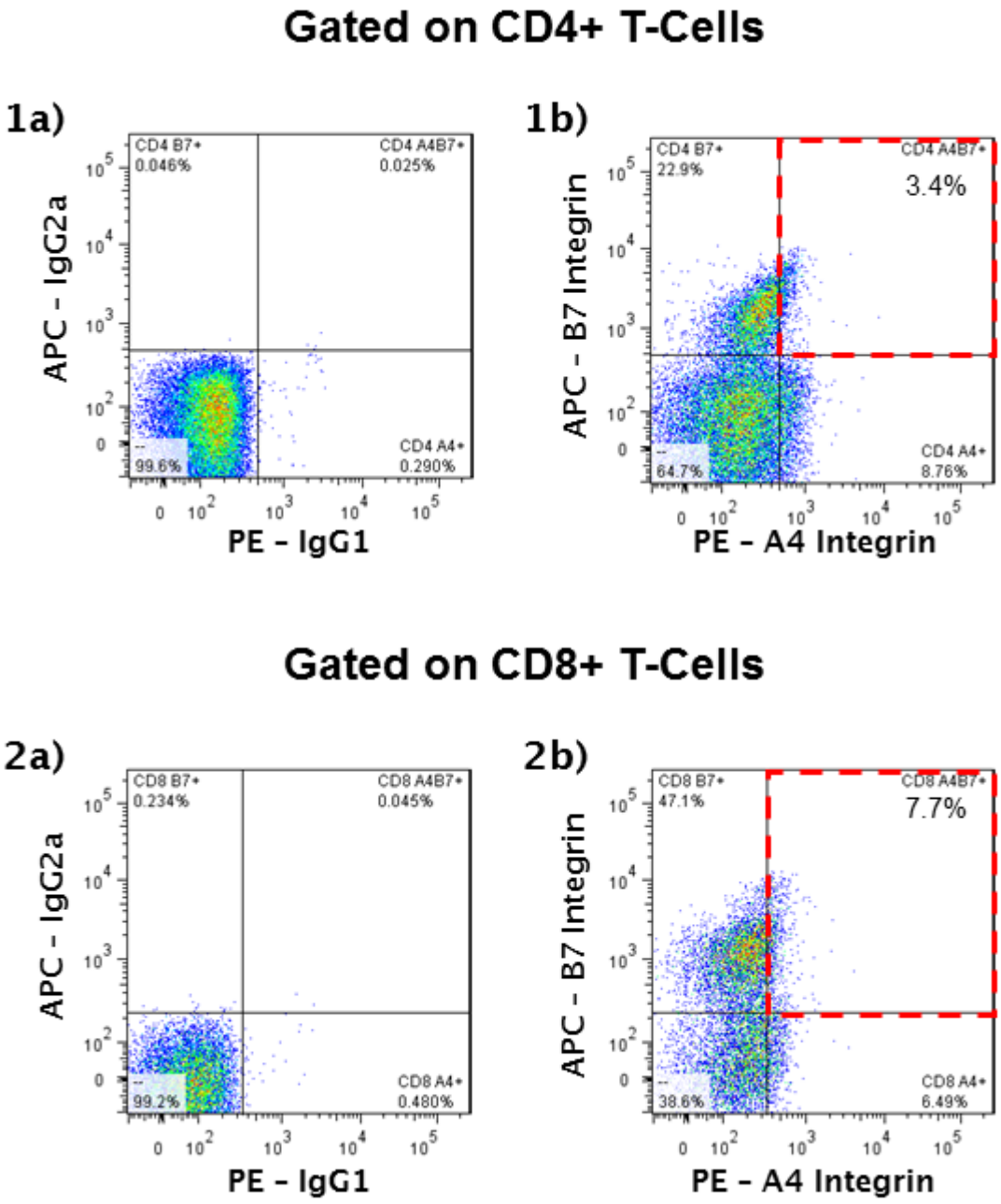


Figure 14 Legend

PBMC were isolated from a healthy blood donor. A representative example is shown; gated on live CD3+ T-cells. Integrin α4β7+ T-cells were identified in CD4+ (upper panel) and CD8+ (lower panel) T-cells. A negative gate was created using matched isotype control antibodies (1a) and (2a). Anti-integrin α4 and anti-integrin β7 antibodies were used to identify positively staining T-cells (1b) and (2b).

Table 16: Results of Peripheral Blood T-Lymphocyte Subset Analysis Using Flow Cytometry

Lymphocyte Subset	Chronic Pancreatitis		Control		P value
	Median	Interquartile Range	Median	Interquartile Range	
CD3+ (% of lymphocytes)	80.45	73.88 - 82.70	74.50	69.43 – 82.03	0.282
CD4+ (% of CD3+)	66.65	56.58 - 71.18	73.10	60.30 - 81.23	**0.076**
CD4+ Memory (% of CD4+)	49.65	33.93 – 61.88	49.90	32.98 - 62.38	0.756
CD4+ Naïve (% of CD4+)	50.35	38.13 - 66.08	50.10	37.63 - 67.03	0.756
CD4+ α4β7+ (% of CD4+)	2.31	1.40 - 3.32	3.39	1.49 - 5.15	0.214
CD8+ (% of CD3+)	23.95	19.48 - 32.53	20.85	12.30 - 29.30	0.130
CD8+ Memory (% of CD8+)	48.05	36.75 - 59.65	49.70	40.40 - 55.93	0.846
CD8+ Naïve (% of CD8+)	51.95	40.35 - 63.25	50.30	44.08 - 59.60	0.846
CD8+ α4β7+ (% of CD8+)	3.90	2.71 - 6.29	7.73	3.23 - 15.15	**0.052**
CD8+ CD161+ (% of CD8+)	5.80	4.13 - 9.33	9.08	5.50 - 13.60	0.126
Double Negative (% of CD3+)	4.96	2.73 - 8.27	4.30	2.30 - 6.33	0.288
CD4 to CD8 ratio (CD4+/CD8+)	2.80	1.72 - 3.65	3.53	2.06 - 6.60	**0.097**

Figure 15: Dot Plots of CD4+ and CD8+ T-Cells in the Peripheral Blood of Chronic Pancreatitis Patients and Controls

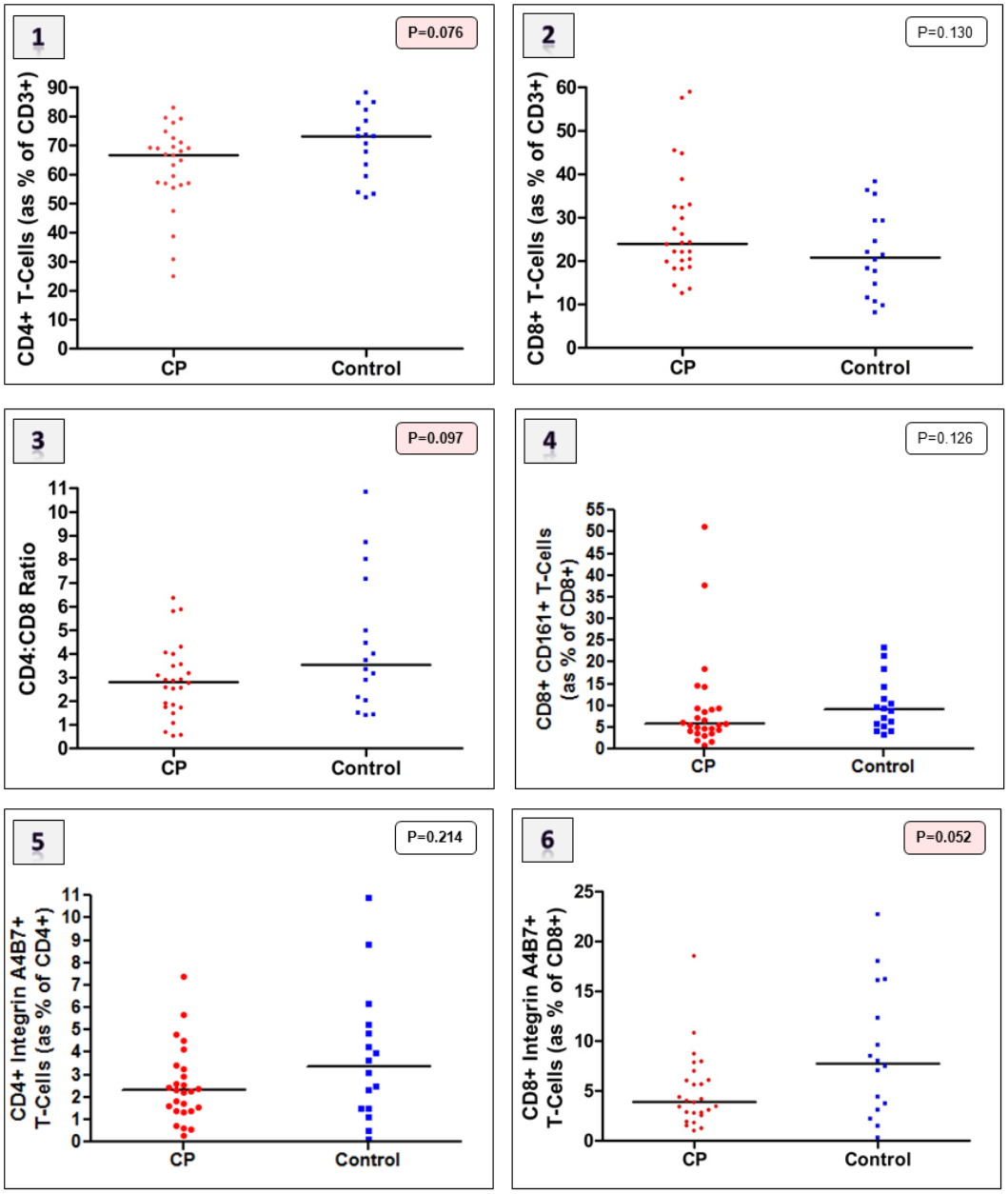


Figure 15 Legend

PBMC from 26 patients with chronic pancreatitis and 16 controls were analysed using flow cytometry. The dot plots show the results of lymphocyte subsets of interest and in particular those that demonstrate a trend towards a significant difference between groups. 1) CD4+ T-cells, 2) CD8+ T-cells, 3) CD4:CD8 ratio, 4) CD8+ CD161+ T-cells, 5) CD4+ integrin α4β7+ T-Cells and 6) CD8+ integrin α4β7+ T-Cells. The black line shows the median for each data set. p values were obtained using a Mann-Whitney U Test.

Conclusion

These results demonstrate that no statistical difference exists between the peripheral blood lymphocyte subsets of CP patients and controls in any of the populations analysed. However a trend towards a lower proportion of CD4+ T-cells in CP patients was noted. A corresponding change in the CD4:CD8 ratio but no associated change in the proportion CD8+ T-cells was also seen. Work by previous authors has been inconclusive as to whether a true change in CD4+ and CD8+ peripheral blood lymphocyte subsets exists: Initial publications demonstrated a reduction in the percentage of CD8+ T-cells in CP patients compared to controls (n=11);[134] but others subsequently found an increase in the numbers of CD4+ and CD8+ T-cells (n=48),[135] a reduction in CD4+ T-cells (n=27),[136] and an increase in the total number of CD4+ T-cells with no change in CD8+ T-cells (n=18).[138] Each of these studies contained fewer participants and therefore are more likely to display statistical error. It is nevertheless difficult to conclude whether the peripheral blood of chronic pancreatitis patients contains a lower proportion of CD4+ and a higher proportion of CD8+ T-cells compared to controls. A very large patient cohort may be able to definitively elucidate if any true statistical difference exists.

Interestingly no difference in the ratio of naïve to memory T-lymphocytes in the blood of CP patients was found. This is contradictory to the findings of Grundsten *et al* who demonstrated an increase in both CD4+ and CD8+ CCR7+CD45RA- central memory T-cells in CP patients compared to healthy controls (n=27).[137] Differences between the populations studied and the techniques used may explain this discordance: Half of their patients had severe disease that necessitated surgery; and they used CCR7 as well as CD45RA to identify peripheral blood memory T-cells. CCR7 is found on central memory T-cells; it allows re-circulation of cells through the spleen and lymph nodes, back into the circulation.[213, 214] I therefore measured all $CD45RO^+$ memory T-cells cells (akin to $CD45RA^-$) rather than the central memory $CCR7^+$ subset. I thus believe that my data represents a true finding that in CD4+ and CD8+ T-cells, no difference in the balance of $CD45RO^+$ memory and $CD45RO^-$ naïve T-cells exists in the peripheral blood of chronic pancreatitis patients.

The most thought-provoking finding was that there was a trend towards a reduction in the proportion of CD8+ integrin α4β7+ T-cells in chronic pancreatitis patients compared to controls. The reason for this is unclear however it may represent pooling of these cells in the inflamed pancreas which leads to a corresponding decrease in those present in peripheral blood. The chemokine and integrin signature of exocrine

pancreas infiltrating lymphocytes has yet to be identified. It is postulated that this would be similar to that for the endocrine pancreas, whereby T-cells that are involved in the pathogenesis of diabetes mellitus in mouse models express integrin α4β7.[121, 123] Integrin α4β7 is expressed on a distinct subset of memory CD4+ and CD8+ T-cells and it directs their trafficking to intestinal sites of inflammation via interactions with mucosal addressin cell adhesion molecule 1 (MAdCAM-1).[115, 116] The site of initial antigen exposure can define the phenotype of the memory population, as CD4+ T-cells primed in intestinal lymphoid organs increase integrin α4β7 expression, whereas those primed in cutaneous lymph nodes rapidly lose integrin α4β7 expression.[215] It would therefore be of great interest to define whether integrin α4β7+ T-cells were found in the pancreatic tissue of CP patients in significant numbers and whether they were primed in pancreatic lymph nodes.

T-LYMPHOCYTE SUBSETS IN PANCREATIC TISSUE

T-lymphocyte subsets were identified in pancreatic tissue using either immunohistochemical analysis of whole tissue or flow cytometry analysis of pancreatic tissue homogenates as previously described. Statistical analysis was undertaken using PASW Statistics 18.0.0 employing a Mann-Whitney U Test.

The tissue of six patients with chronic pancreatitis (5 male, 1 female, median age 54 years, age range 35-75 years) (See Table 13: P04, P13, P19, P29, P44, P46) and four control patients (1 male, 3 female, median age 63 years, age range 47 – 75 years) (See Table 14: P07, P10, P12, P45) was examined using immunohistochemistry. Representative examples of negative control haematoxylin staining of normal pancreatic and chronic pancreatitis tissue is shown in Figure 16; T-cell staining with anti-CD3 antibodies in Figure 17; and CD4 and CD8 T-cell staining in Figure 18. Pancreatic tissue from eight patient with chronic pancreatitis (all male, median age 46.5 years, age range 35-63 years) (See Table 13: P04, P13, P19, P23, P29, P34, P44, P46) and three controls (2 male, 1 female, median age 68 years, age range 62-73 years) (See Table 14: P26, P40, P45) was enzymatically and mechanically digested to allow the isolation of mononuclear cells, which were then cryopreserved before analysis using flow cytometry (See Figure 19).

A significantly higher number of CD3+ T-cells were identified in the pancreatic tissue of CP patients compared to controls when analysed using immunohistochemistry (CP 6.52 cells/mm^2 *vs.* 1.68 cells/mm^2 control; p=0.033) (See Table 17 and Figure 20). The analysis of CD3+ T-cells in pancreatic tissue using flow cytometry is not possible as the results are expressed as a proportion of the cells in the superior hierarchy i.e. in the lymphocyte gate. As these proportions are influenced by other lymphocytes present no comparison between disease and control groups can be made.

Interestingly, whilst no difference in the number of CD4+ and CD8+ T-cells was detected when the pancreatic tissue of CP patients and controls was analysed using immunohistochemistry, a significant difference was detected when they were assessed using flow cytometry (See Table 17, Table 18 and Figure 20). In particular it should be noted that when pancreatic tissue homogenates from control patients were assessed using flow cytometry, very few CD3+ T-cells were detected in the lymphocyte gate and none of these convincingly also stained for CD4 or CD8. The flow cytometry dot plots displaying CD4, CD8 and DN T-cell subset results from the pancreatic tissue of all chronic pancreatitis patients are shown in Figure 19.

Figure 16: GMA Embedded Pancreatic Tissue Stained with Haematoxylin

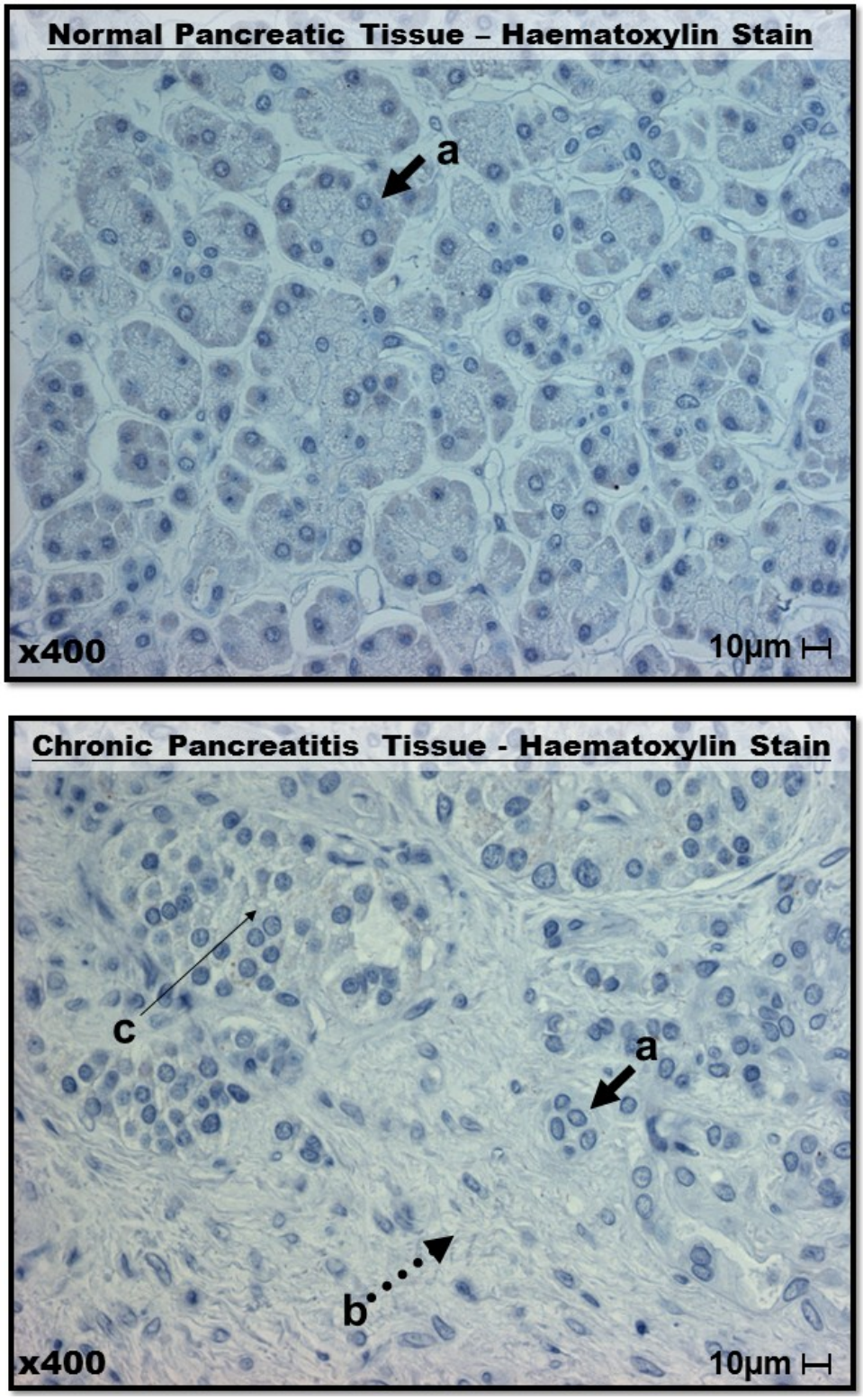


Figure 16 Legend

Haematoxylin stained normal pancreatic tissue (top image) containing numerous acini (a) is shown. In chronic pancreatitis tissue (bottom image) acini (a) are lost, and those that survive are contained within fibrous stroma (b). Islets (c) are spared until fibrosis is extensive and disease advanced.

Figure 17: CD3+ T-Cells in GMA Embedded Pancreatic Tissue

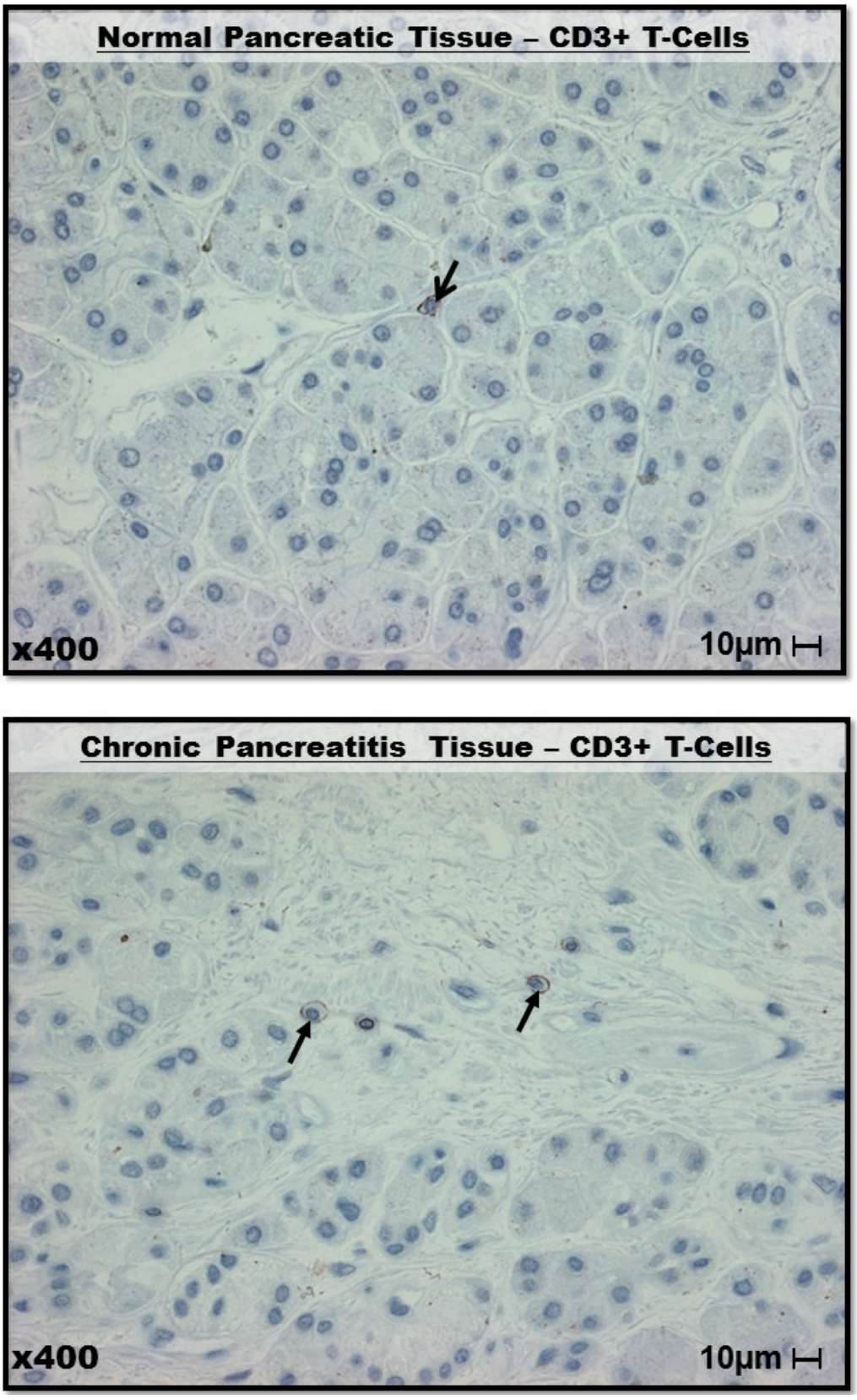


Figure 17 Legend

CD3+ T-cells (arrowed) are identified in normal (upper image) and chronic pancreatitis tissue (lower image) by red cell membrane ring staining.

Figure 18: CD4+ and CD8+ T-Cells in GMA Embedded Chronic Pancreatitis Tissue

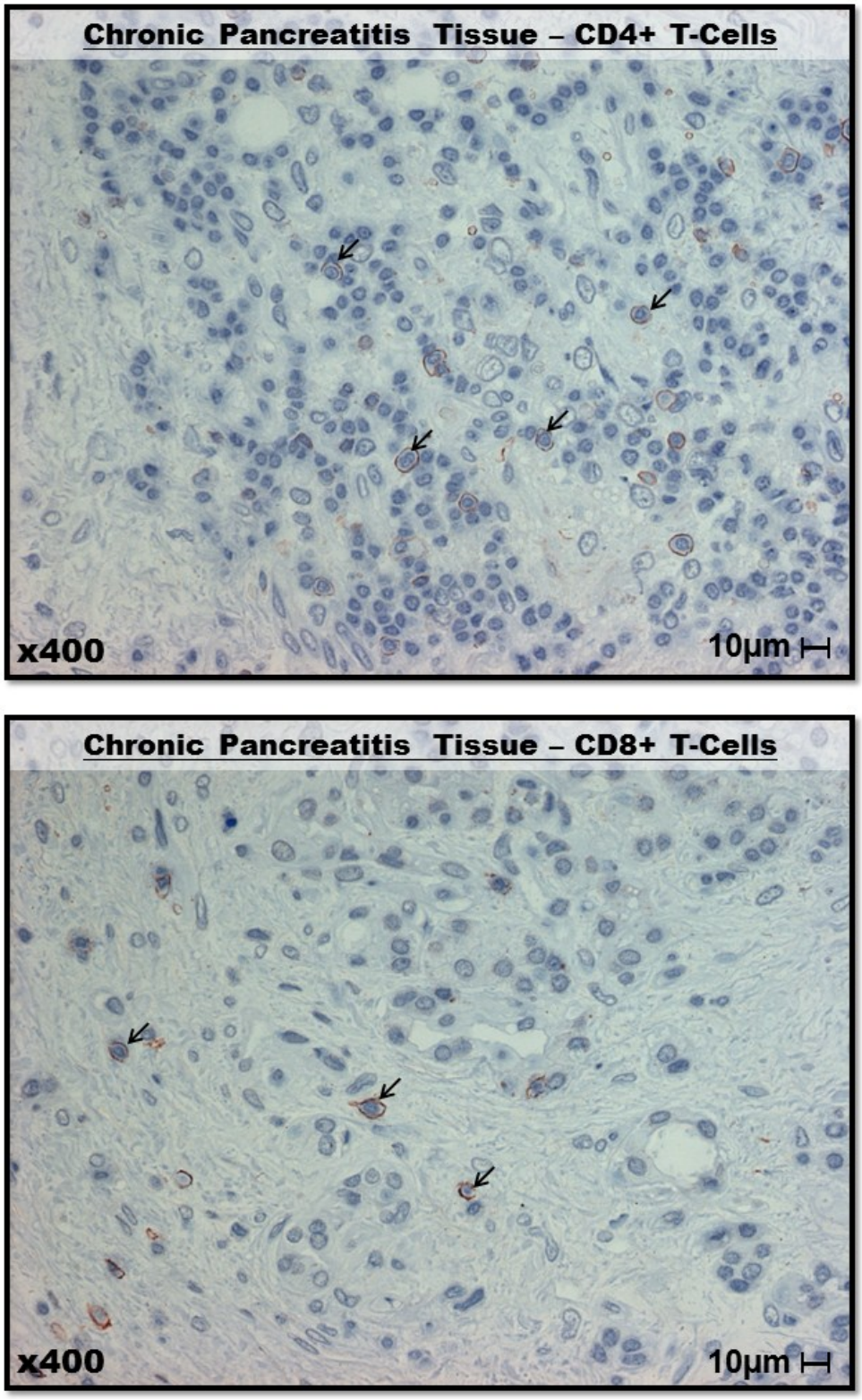


Figure 18 Legend

CD4+ (upper image) and CD8+ (lower image) T-cells (arrowed) are identified in chronic pancreatitis tissue by red cell membrane ring staining. Not all positively stained cells are arrowed.

Figure 19: T-Cell Subsets Identified in Chronic Pancreatitis Tissue Using Flow Cytometry

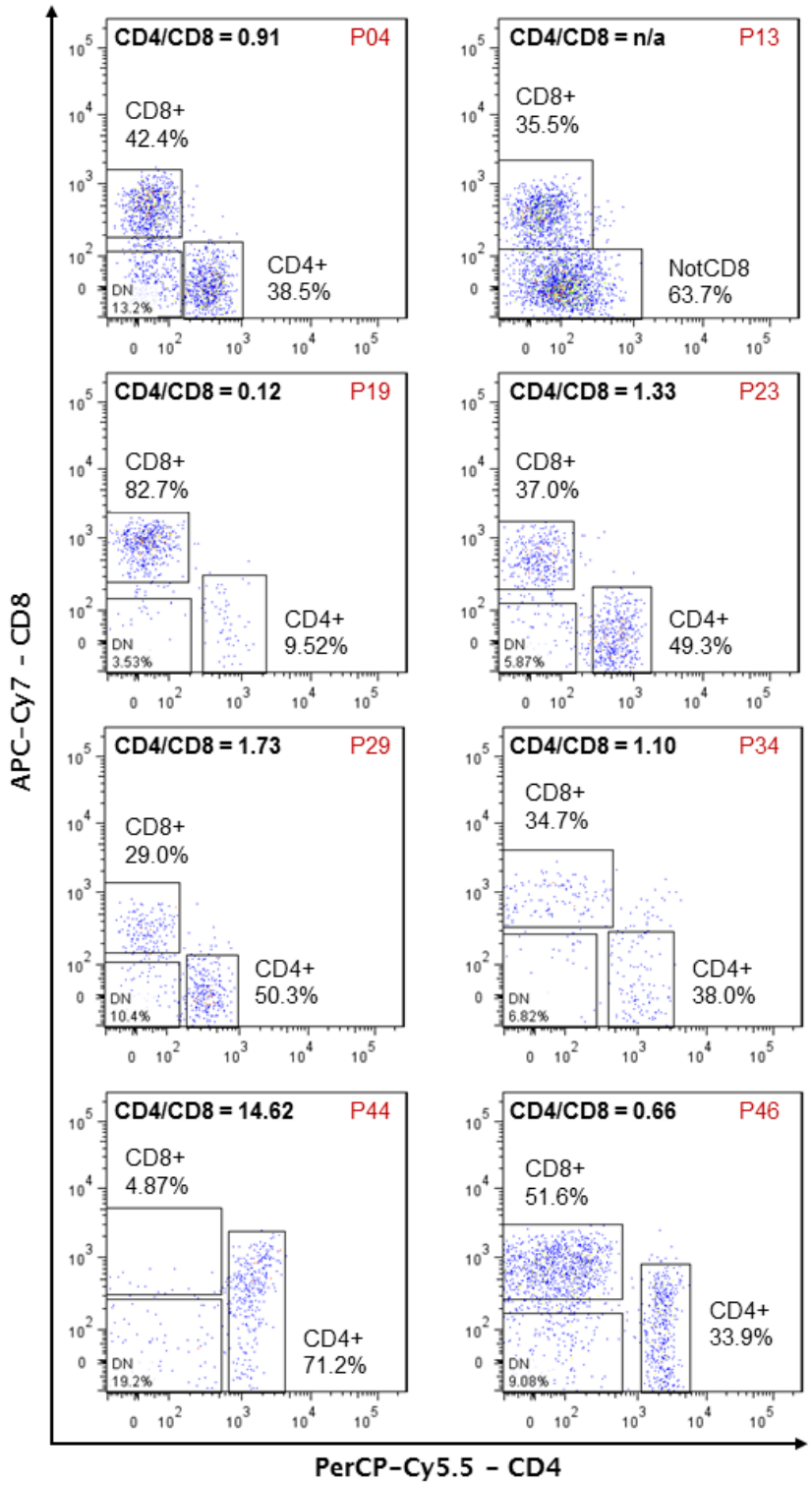


Figure 19 Legend

The pancreatic tissue from eight patients with chronic pancreatitis was disaggregated and analysed using flow cytometry. The dot plots for each patient are shown (patient identifier shown in red – See Table 13); gated on live CD3+ T-cells.

Table 17: Results of Immunohistochemical Analysis of Pancreatic Tissue

Lymphocyte Subset	Chronic Pancreatitis		Control		P value
	Median	Interquartile Range	Median	Interquartile Range	
CD3+ (per mm^2)	6.52	4.42 - 32.27	1.68	0.18 - 2.80	**0.033**
CD4+ (per mm^2)	0.77	0.24 - 8.89	0.34	0.06 - 0.47	0.136
CD8+ (per mm^2)	3.66	0.92 - 23.44	2.16	0.12 - 6.22	0.394
CD4:CD8	0.42	0.17 - 0.59	0.08	0.01 - 0.78	0.286

Table 18: Result of Flow Cytometry Analysis of Pancreatic Tissue

Lymphocyte Subset	Chronic Pancreatitis (Median)	Control (Median)	P value
CD4+ (% of CD3+)	38.5	0	**0.015**
CD8+ (% of CD3+)	34.8	0	**0.013**
Double Negative (% of CD3+)	9.08	0	**0.015**

Figure 20: Dot Plots Showing Results of Immunohistochemical and Flow Cytometry Analysis of Pancreatic Tissue

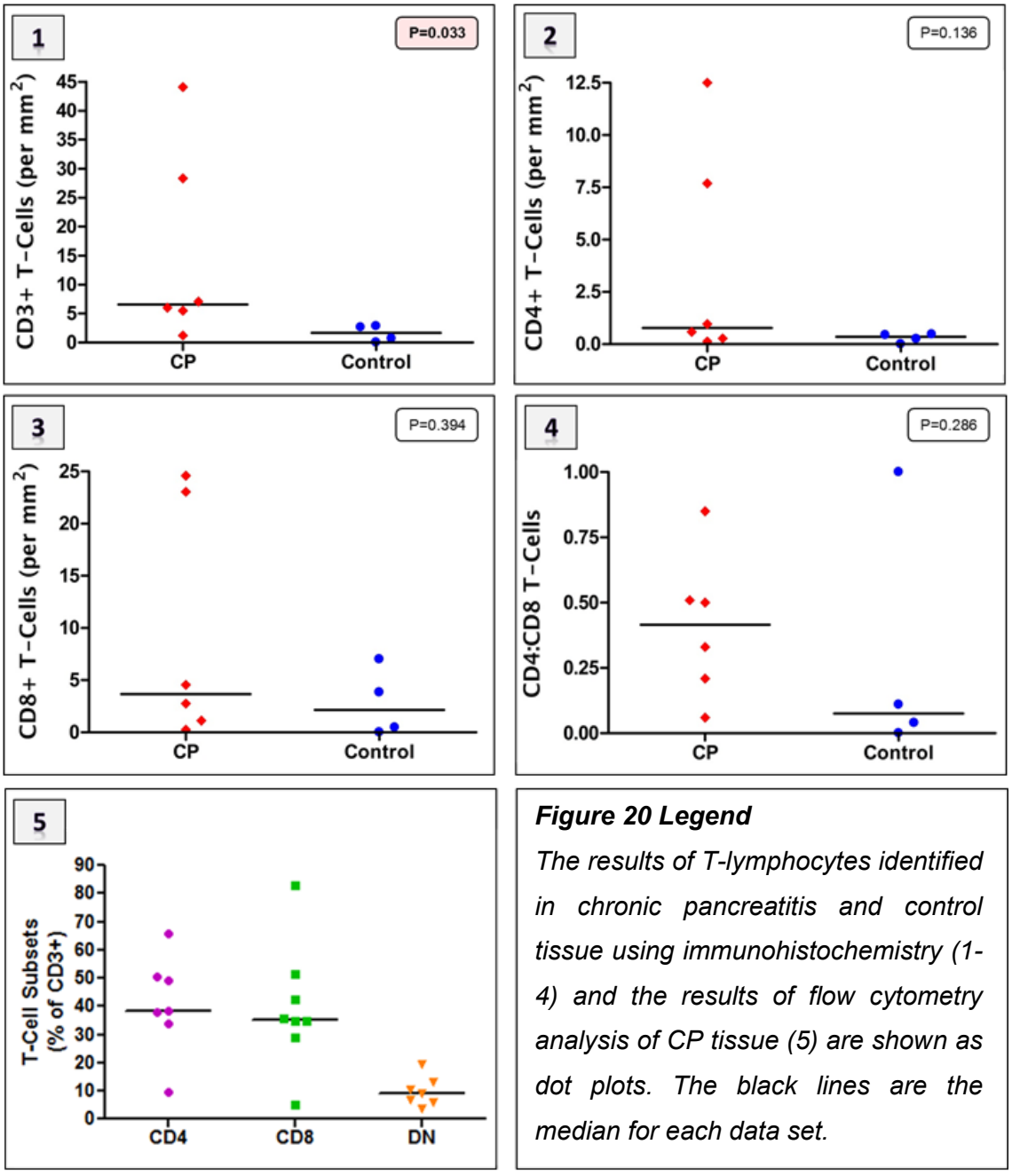


Figure 20 Legend

The results of T-lymphocytes identified in chronic pancreatitis and control tissue using immunohistochemistry (1-4) and the results of flow cytometry analysis of CP tissue (5) are shown as dot plots. The black lines are the median for each data set.

Conclusion

It is clear that the number of CD3+ T-cells is higher in CP tissue compared to controls as previously described by many authors. However it is interesting that despite the distinctly higher number of CD3+ T-cells in GMA embedded CP tissue I found no difference in the number of CD4+ or CD8+ T-cells present. Nevertheless pancreatic tissue assessed using flow cytometry did reveal significant differences between CP and control groups. The reasons for these discrepancies may be explained in a number of possible ways:

1. **Potential problems with immunohistochemical staining of GMA embedded pancreatic tissue**

GMA embedded human tonsillar tissue is stained reproducibly with anti-CD3, -CD4 and -CD8 antibodies. However whilst intrapancreatic CD3+ T-cells are clearly identifiable and stain well, it may be that CD4 and CD8 T-cells stain less well. To examine this further calculations of T-cell numbers can be made. If the number of CD4+ T-cells is added to the number of CD8+ T-cells, and the total subtracted from the total number of CD3+ T-cells [$CD3^+$ - ($CD4^+$ + $CD8^+$)] for CP and control tissue the results are as follows:

Chronic Pancreatitis		**Control**	
Patient	CD3 – (CD4+CD8)/mm^2	Patient	CD3 – (CD4+CD8)/mm^2
P04	13.33	P07	-0.24
P13	0.81	P10	-4.41
P19	2.21	P12	-1.64
P29	2.15	P45	0
P44	3.90		
P46	-8.76		

Furthermore comparison of the CD4:CD8 ratio in CP tissue using both immunohistochemistry and flow cytometry reveals the following:

CP Patient	CD4:CD8 Immunohistochemistry	CD4:CD8 Flow Cytometry
P04	0.33	0.91
P13	0.50	n/a
P19	0.06	0.12
P29	0.21	1.73
P44	0.85	13.53
P46	0.51	0.66

It is unlikely that the discrepancies noted in chronic pancreatitis tissue are related to the presence of significant numbers of double negative T-cells as this result was not found when CP tissue was examined using flow cytometry. In addition the CD4:CD8 ratios vary significantly according to the method used.

These results could therefore be explained by:

- Poor CD4 staining in pancreatic tissue – Immunohistochemical analysis of CD4+ T-cells has historically been a difficult process as the antibodies can produce patchy results. Whilst the positive control staining for each sample was good in all cases, in could be that pancreatic tissue stains less well.
- Tissue sectioning – lymphocytes are approximately 10-20μm in diameter and tissue sections are 2μm thick. It is possible therefore that whilst some sections will stain a particular CD3+ T-cell the other sections may not include the same cell. This problem is inherent to all immunohistochemical studies.
- The sample size is small and number of slides stained is small – the low number of cases included coupled with the assessment of two slides for each cell marker for each case may not generate enough data to prevent statistical error. The assessment of two slides per cell marker per case is a well-established method thus increasing the number of participants would be the only means of avoiding this problem.
- Patchy inflammation in chronic pancreatitis causes aberrant results – review of the raw data (as shown in Figure 20) reveals that only in some pieces of chronic pancreatitis tissue analysed are significant numbers of T-cells seen. Sampling error may therefore adversely affect the results. In particular the immunohistochemistry data suggests that CP involves infiltration of more CD8+ than CD4+ T-cells, but the flow cytometry data suggests that they are found in equal proportions. A larger sample size might resolve these problems.

2. Potential problems with flow cytometry analysis of pancreatic tissue

Whilst CD3+, CD4+ and CD8+ T-cells were detected in normal pancreatic tissue using immunohistochemistry, very few T-cells were detected using flow cytometry. As the absolute number of T-cells is not known when pancreatic tissue is analysed using flow cytometry, but rather the proportion of T-cells found in the lymphocyte gate, comparison between disease and control groups is not possible. In normal pancreatic tissue no CD3+ T-cells stained for CD4 or CD8. It is not feasible that all of these CD3+ T-cells are double negative T-cells as this supposition is not supported by the

immunohistochemistry data. I therefore conclude that either the numbers of T-cells detected in normal pancreatic tissue are so low that there is no convincing population seen thus the CD4 and CD8 staining appears spurious; or that T-cells and T-cell antigens are lost when the tissue is digested and attempts to isolate mononuclear cells are undertaken. These effects become more pronounced if the tissue contains very low numbers of lymphocytes.

In conclusion whilst immunohistochemistry can sensitively detect a rare event it requires a lot of tissue. Flow cytometry on the other hand is less sensitive but can assess more tissue and becomes increasingly sensitive when a critical number of cells are detected. It is likely that the low numbers of patients in each group compounds these inherent difficulties. Nevertheless the data suggest that CD3+ T-cells are significantly enriched in chronic pancreatitis tissue. The proportion of CD4 to CD8 T-cells in chronic pancreatitis is likely to be 1:1 as shown by flow cytometry. This technique is a more powerful means of assessing the proportions of T-cell subsets in the tissue analysed and it avoids the potential problems associated with patchy pancreatic inflammation. Previously published immunohistochemical analysis has revealed disagreement as to whether equal numbers of CD4+ and CD8+ T-cells are present, whether CD4+ T-cells are predominant, or whether CD8+ T-cells are more prevalent.[39, 130-132] However published flow cytometry analysis of CP tissue has found equal numbers of CD4+ and CD8+ T-cells.[133] On balance therefore I believe that my results appropriately reflect the inherent difficulties faced with examining pancreatic tissue and that the T-cell infiltrate found in chronic pancreatitis tissue is most likely equally composed of both CD4 and CD8 T-cells as my flow cytometry data suggests.

PAIRED ANALYSIS OF T-LYMPHOCYTE SUBSETS IN PERIPHERAL BLOOD AND PANCREATIC TISSUE USING FLOW CYTOMETRY

PBMC and pancreatic infiltrating mononuclear cells from eight CP patients (all male, median age 46.5 years, age range 35-63 years) (See Table 13: P04, P13, P19, P23, P29, P34, P44, P46) were analysed using flow cytometry to identify T-cell subsets as previously described. Paired statistical analysis was performed using the Wilcoxon Signed Ranks Test.

The proportion of CD4+ T-cells is significantly lower in the pancreatic tissue of patients with CP compared to peripheral blood (median tissue *vs.* blood: 38.5% *vs.* 60.6%; p=0.03) (See Table 19 and Figure 21). However no difference existed when CD8+ and DN T-cells were examined in the same manner. The flow cytometry dot plots for all patients' tissue and blood, displaying CD4+, CD8+ and DN T-cells, are shown in Figure 22.

Table 19: Results of Paired Analysis of Peripheral Blood and Pancreatic Tissue T-Cells in Patients with Chronic Pancreatitis

Lymphocyte Subset	Blood		Tissue		P value
	Median	Interquartile Range	Median	Interquartile Range	
CD4+ (% of CD3+)	60.60	40.28 - 75.48	38.50	33.90 - 50.30	**0.03**
CD8+ (% of CD3+)	29.15	19.20 - 51.73	35.15	30.43 - 49.30	0.33
DN (% of CD3+)	4.845	3.03 - 5.89	9.08	5.87 - 13.20	0.13
CD4:CD8 Ratio	2.09	0.89 - 3.85	1.10	0.66 - 1.73	0.40

Figure 21: Dot Plots Showing Paired Results of Peripheral Blood and Pancreatic Tissue CD4+, CD8+ and Double Negative T-Cells in Chronic Pancreatitis

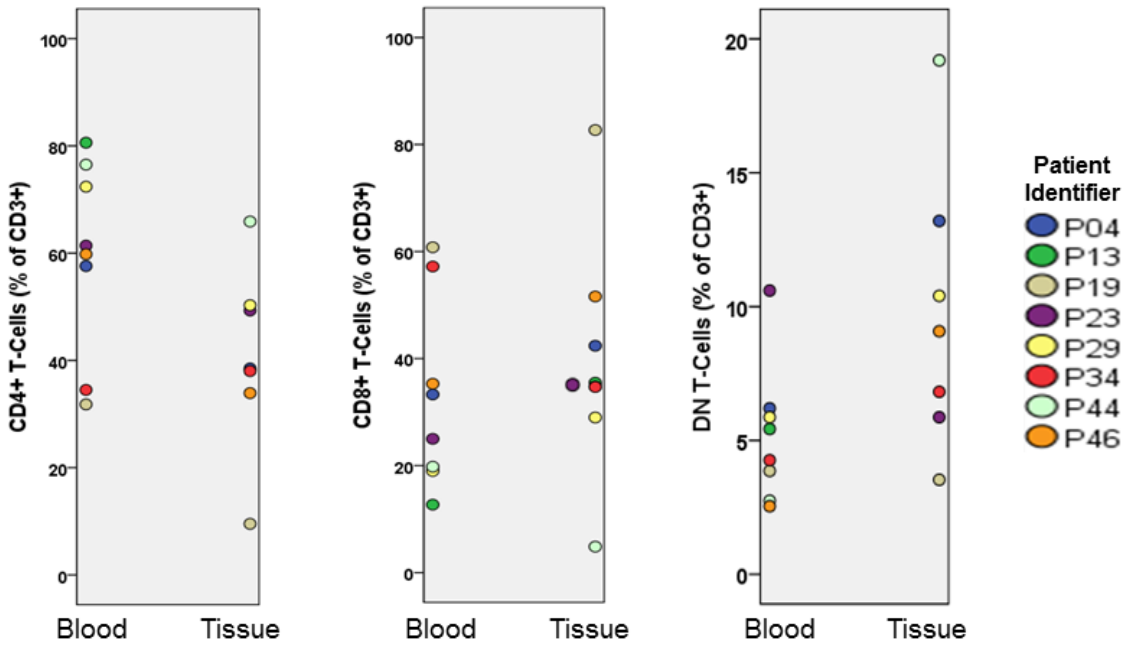


Figure 21 Legend

Blood and pancreatic tissue from eight patients with CP (See Table 13: Demographics of Chronic Pancreatitis Patients) was analysed using flow cytometry. The proportion of CD4+ T-cells (as a percentage of CD3+) is significantly lower in pancreatic tissue (median 38.5%) when compared to blood (median 60.6%) (p=0.03). However there is no difference in the proportion of CD8+ and double negative (DN) T-cells. Note: P13 tissue sample was over-digested resulting in loss of CD4+ staining (See Figure 22).

Figure 22: T-Cell Subsets Identified in Paired Chronic Pancreatitis Tissue and Blood Samples Using Flow Cytometry

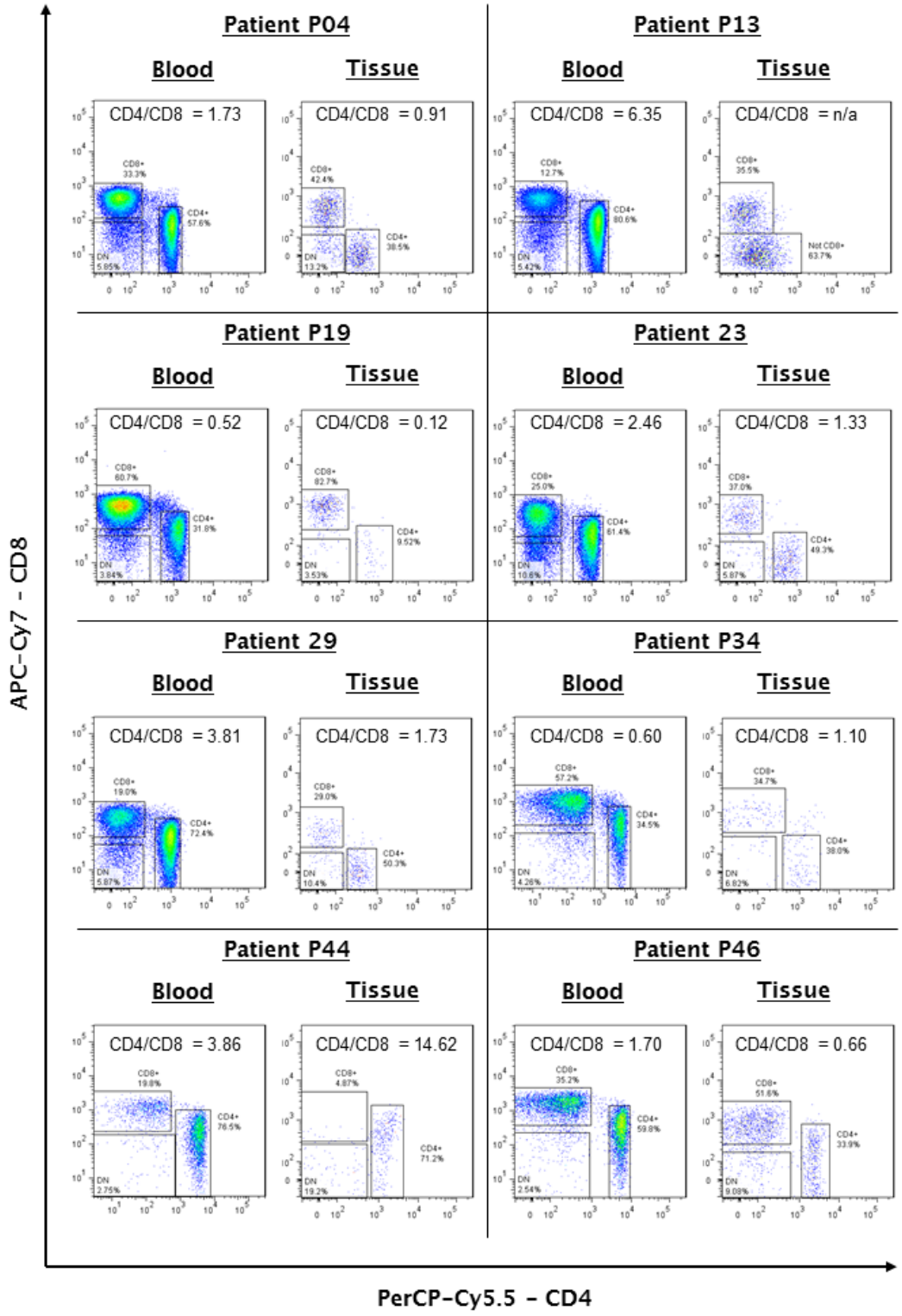


Figure 22

Pancreatic tissue and peripheral blood from eight patients with chronic pancreatitis (See Table 13) was analysed using flow cytometry. Results of paired samples from each patient are shown; gated on live CD3+ T-cells.

Conclusion

Only one published study has previously assessed both pancreas infiltrating and peripheral blood lymphocytes.[139] However this work did not specifically analyse T-cell subsets but instead focused on Treg cells. My data demonstrate a significant reduction in the proportion of CD4+ T-cells that are found in CP tissue compared to blood. Interestingly a corresponding increase in the proportions of CD8+ and DN T-cells present in tissue was not found, nor an alteration in the CD4:CD8 ratio. This is likely explained by an insufficient sample size causing statistical error: Six of the seven patients had a lower proportion of CD4+ in tissue compared to blood (note one patient excluded as CD4 population not clearly defined), creating a statistically significant result; whereas six of eight and five of seven patients had higher proportions of CD8+ and DN T-cells in tissue compared to blood, which were not statistically significant but clearly suggestive of a trend. Expansion of this study in the future would likely produce the expected result of an increase in CD8+ and DN T-cells.

The finding of significant numbers of DN T-cells in pancreatic tissue is intriguing as they are a poorly defined T-cell subset, which includes “unconventional” lymphocytes such as mucosal-associated invariant T-cells (MAIT), invariant natural killer T-cells and γδ T-cells. These discrete subpopulations tend to be evolutionarily conserved, localise to specific tissues, and are often considered to act at the frontier between innate and adaptive immunity: like adaptive immune cells they express T-cell receptors, but like innate immune cells, they tend to recognise a limited set of antigens and respond quickly to an immune challenge.[216] Recent evidence suggests that they contribute significantly to the development and establishment of acute and chronic inflammatory diseases, and that they play a potential pathogenic role in autoimmunity as well as suppressing excessive immune responses which may be deleterious to the host.[217] Therefore their further investigation is clearly merited in the context of chronic pancreatitis. The examination of pancreatic tissue and peripheral blood for MAIT cells, an area which has never been investigated before, was thus performed. The results of this analysis can be seen on page 143.

T-HELPER AND T-REGULATORY LYMPHOCYTES IN PERIPHERAL BLOOD

Cryopreserved PBMC isolated from chronic pancreatitis patients and controls were thawed and analysed using flow cytometry as previously described. PBMC for T-helper cell analysis were cultured overnight and then stimulated for five hours using PMA and ionomycin before analysis. The blood of 15 patients with CP (all male, median age 48.2 years) and 14 controls (10 male, 4 female, median age 56.1 years) was assessed (See Table 20). Th1, Th2 and Th17 cells were detected as having positive staining for IFN-γ, IL-13 and IL-17 respectively (See Figure 23); and Treg cells were characterised by expression of CD25 and FoxP3, with low or no CD127 expression (See Figure 24). Statistical analysis was undertaken using PASW Statistics 18.0.0 employing a Mann-Whitney U Test.

A significantly higher percentage of IFN-γ^+ Th1 cells (CP 15.2% *vs.* 8.11% control; p=0.03), IL-13^+ Th2 cells (CP 2.00% *vs.* 1.17% control; p=0.03), IL-17^+ Th17 cells (CP 1.23 *vs.* 0.41% control; p=0.003), dual secreting IFN-γ^+ IL-17^+ Th17 cells (CP 0.11% *vs.* 0.03% control; p=0.003) and $CD25^+FoxP3^+$ Treg cells (CP 6.30% *vs.* 4.40% control; p=0.05) were found in the peripheral blood of patients with chronic pancreatitis compared to controls. No difference was found in the ratio of Th1:Th2 cells or the percentage of activated $CD4^+CD25^+$ T-cells in each patient group, although a trend towards a decrease in the Treg:Th17 ratio was suggested in CP compared to controls (CP 5.46 *vs.* 10.49 control; p=0.15) (See Table 21 and Figure 25). The dot plots of each patient's peripheral blood flow cytometry analysis identifying Th1, Th2, Th17 and Treg cells can be seen in Figure 41, Figure 42 and Figure 43 in the Appendix.

Patients with the highest proportion of Th1 cells tended to have high proportions of Th2 and Treg cells (See Table 23). Furthermore individuals who consume excess alcohol (>21/>14 units per week male/female) have significantly more Th1 cells in their peripheral blood than non-drinkers (23.7% vs. 9.81%; p=0.01) (See Table 22 and Figure 25). Chronic pancreatitis patients who continue to consume excess alcohol also have significantly more Th1 cells than non-drinkers (23.7% vs. 10.6%; p=0.05). Smoking appeared to have no effect on proportions of Th-cells or Treg cells.

Table 20: Patient Demographics of Peripheral Blood T-Helper and T-Regulatory Lymphocytes Analysed Using Flow Cytometry

	Chronic Pancreatitis	Control	
Total Number	15	14	
Gender	15 Male	10 Male / 4 Female	
Median Age (years)	47.0	56.0	56.0 M 54.5 F
Age Range (years)	25 - 74	39 - 75	39 – 73 M 42 – 75 F

Table 20 Legend

The specific demographic details for each chronic pancreatitis patient (P02, P04, P11, P13, P14, P15, P16, P18, P20, P21, P24, P25, P28, P35, P44) and control patient analysed (P07, P12, P22, P26, P40, P41, P43, P45, P47, P48, P49, P50, P51, P52) can be seen in Table 13 and Table 14 respectively.

Figure 23: Identification of T-Helper Lymphocytes in Peripheral Blood Using Flow Cytometry

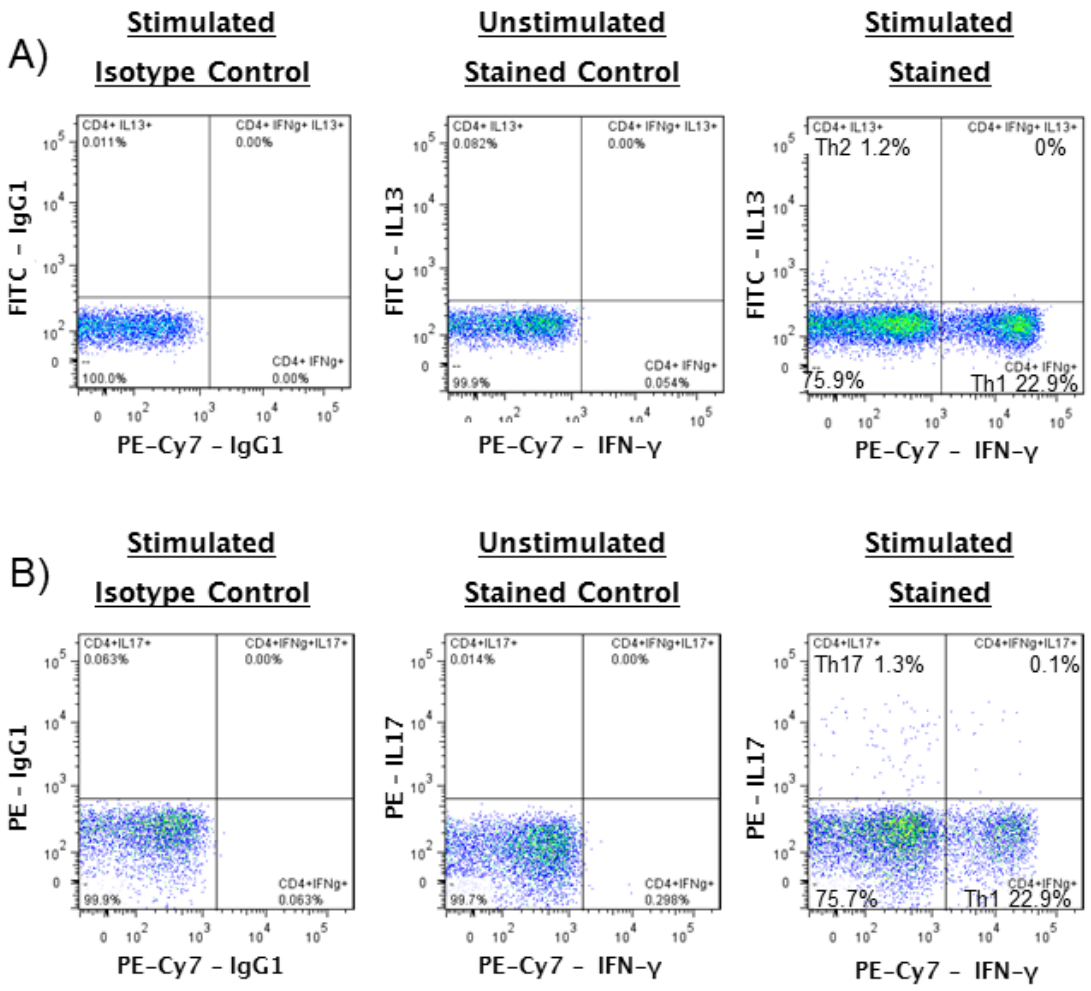


Figure 23 Legend

PBMC were isolated from a patient with chronic pancreatitis, stimulated with PMA and ionomycin, stained for T-cell surface antigens and intracellular cytokines, and analysed using flow cytometry. The results of a representative patient are displayed; gated on live CD4+ T-cells. Control experiments are shown in the left and middle panels: stimulated cells stained with matched isotype control antibodies; and unstimulated cells stained for intracellular cytokines. Stimulated and stained cells are shown in the right hand panels for A) IFN-γ^+ Th1 and IL-13^+ Th2 cells and B) IFN-γ^+ Th1 and IL-17^+ Th17 cells, including dual secreting IFN-γ^+IL-17^+ cells.

Figure 24: Identification of Peripheral Blood T-Regulatory Lymphocyte Using Flow Cytometry

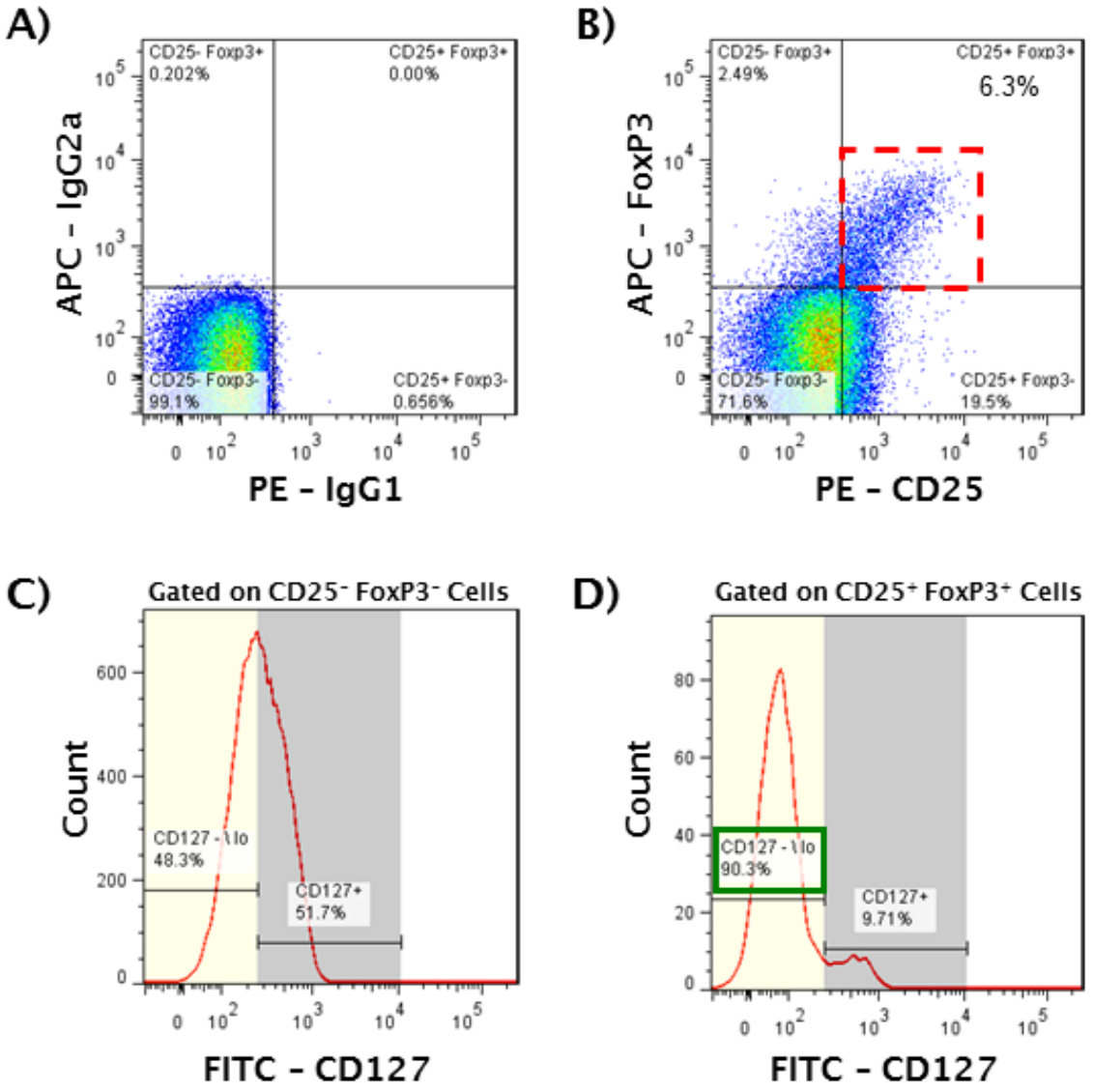


Figure 24 Legend

PBMC were isolated from a patient with chronic pancreatitis, fixed and permeabilised, stained with antibodies to cell membrane components and transcription factors, and analysed using flow cytometry. Results from a representative patient are displayed. (A) Isotype control and (B) $CD25^{+}FoxP3^{+}$ Treg cells, gated on live CD4+ T-cells, are shown. (C) Half of $CD25^{-}FoxP3^{-}$ cells are $CD127^{lo/-}$; whereas (D) over 90% of $CD25^{+}FoxP3^{+}$ Treg cells are $CD127^{lo/-}$ confirming their phenotype.

Table 21: Results of Peripheral Blood T-Helper and T-Regulatory Lymphocyte Analysis Using Flow Cytometry

T-Lymphocyte Lineage	Chronic Pancreatitis		Control		P value
	Median	Interquartile Range	Median	Interquartile Range	
Th1 (% of CD4+)	15.20	9.25 - 22.80	8.11	5.75 - 13.73	**0.03**
Th2 (% of CD4+)	2.00	1.21 - 3.01	1.17	0.74 - 1.94	**0.03**
Th17 (% of CD4+)	1.23	0.57 - 1.68	0.41	0.29 - 0.65	**0.003**
Treg (% of CD4+)	6.30	4.10 - 7.59	4.40	3.78 - 4.91	**0.05**
IFN-γ^+ IL-17$^+$ (% of CD4+)	0.11	0.05 - 0.24	0.03	0.007 - 0.05	**0.003**
Th1:Th2	6.97	4.63 - 12.77	6.33	4.75 - 12.15	0.93
Treg:Th17	5.46	2.92 - 12.88	10.49	5.98 - 13.24	0.15
CD25+ (% of CD4+)	19.66	15.07 - 29.40	19.41	12.92 - 22.03	0.38

Table 22: Results of Peripheral Blood Th1 Analysis in Drinkers and Non-Drinkers

Patient Group	Drinker		Non-Drinker		P value
	Median	Interquartile Range	Median	Interquartile Range	
All Participants	23.70	16.78 – 31.45	9.81	6.48 – 16.85	**0.01**
Chronic Pancreatitis	23.70	16.78 – 31.45	10.60	7.45 – 20.7	**0.05**

Figure 25: Dot Plots Showing Flow Cytometry Analysis of Peripheral Blood T-Helper and T-Regulatory Lymphocytes in Chronic Pancreatitis Patients and Controls

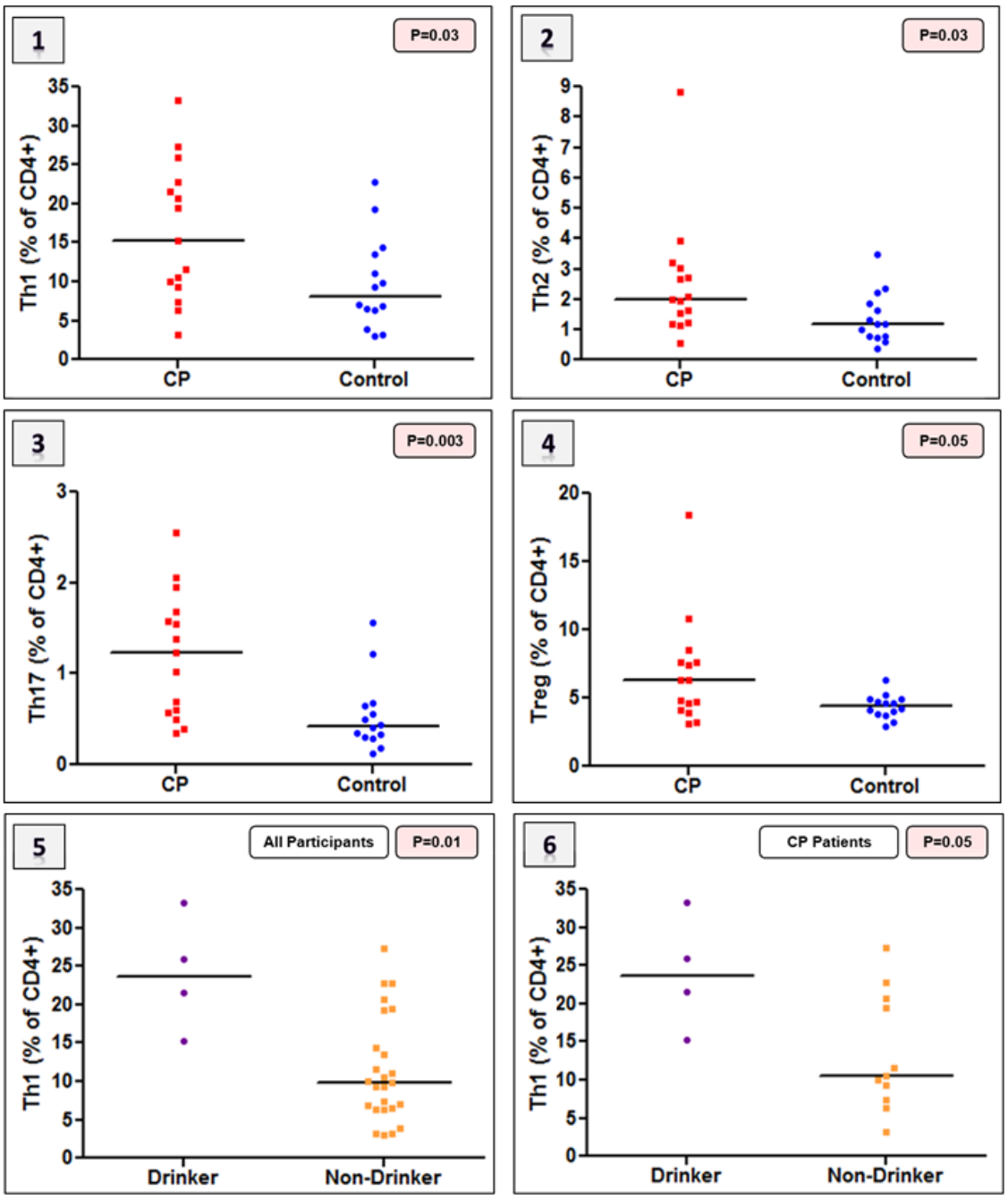


Figure 25 Legend

CD4+ T-helper and T-regulatory lymphocytes were identified in the peripheral blood of 15 patients with chronic pancreatitis and 14 controls. 1) IFN-γ+ Th1 cells, 2) IL-13+ Th2 cells, 3) IL-17+ Th17 cells, and 4) CD25+FoxP3+ T-regulatory cells are shown. Th1 cells in 5) all participants, divided into drinkers and non-drinkers, and 6) Th1 cells in CP patients who are drinkers and non-drinkers are shown. The black lines show the median for each data set.

Table 23: Individually Ranked Data for T-Helper and T-Regulatory Cells in Blood

Patient ID	Age	Gender	Diagnosis	Alcohol	Still Drinking	Smoker	Th1	Th2	Th17	Treg
P11	74.00	M	CP	Y	Y	Y	33.30	3.90	.70	7.59
P15	51.00	M	CP	Y	N	Y	27.40	8.84	2.06	18.40
P14	41.00	M	CP	Y	Y	Y	25.90	2.00	.39	6.35
P4	35.00	M	CP	Y	N	Y	22.80	1.15	1.68	3.87
P22	45.00	M	N	Y	N/A	N	22.80	1.18	1.56	3.67
P35	32.00	M	CP	Y	Y	Y	21.50	3.01	2.56	7.40
P28	51.00	M	CP	Y	N	Y	20.70	3.18	.57	3.17
P24	25.00	M	CP	Y	N	Y	19.50	2.10	1.23	4.10
P12	47.00	F	N	N	N/A	N	19.30	.75	1.21	6.36
P25	32.00	M	CP	Y	Y	N	15.20	1.19	1.02	3.11
P50	42.00	F	N	N	N/A	N	14.40	2.19	.56	4.58
P49	60.00	M	N	N	N/A	N	13.50	.98	.35	4.09
P21	66.00	M	CP	Y	N	N	11.50	2.64	1.58	4.62
P47	61.00	M	N	N	N/A	N	11.00	1.85	.40	4.89
P20	67.00	M	CP	Y	N	Y	10.60	1.52	1.39	7.59
P16	47.00	M	CP	N	N/A	N	10.00	1.95	1.95	4.78
P52	39.00	M	N	N	N/A	Y	9.81	1.61	.19	4.96
P13	39.00	M	CP	Y	N	Y	9.25	.54	.49	6.30
P26	68.00	M	N	N	N/A	Y	9.25	1.16	.65	3.20
P18	61.00	M	CP	N	N/A	N	7.45	1.61	.60	8.56
P41	48.00	M	N	N	N/A	Y	6.97	.60	.49	5.24
P45	62.00	F	N	N	N/A	N	6.91	3.48	.68	4.22
P7	75.00	F	N	N	N/A	N	6.57	2.36	.43	4.02
P2	39.00	M	CP	Y	N	Y	6.38	1.21	1.55	4.67
P48	52.00	M	N	N	N/A	N	6.37	1.30	.30	3.82
P51	47.00	M	N	N	N/A	N	3.89	.79	.13	4.62
P43	67.00	M	N	N	N/A	N	3.17	.35	.28	2.88
P44	63.00	M	CP	Y	N	Y	3.10	2.69	.35	10.80
P40	73.00	M	N	N	N/A	N	3.07	.72	.33	4.67

Table 23 Legend

The results for all chronic pancreatitis and control patients' T-helper and T-regulatory cells is shown ranked for Th1 cells.

Conclusion

It is interesting to find that all the T-helper cell subsets analysed, as well Treg cells, were more prevalent in the peripheral blood of CP patients compared to controls. Only three previously published studies have analysed T-helper and T-regulatory cells in the peripheral blood of CP patients. One early study demonstrated that peripheral blood CD3+ T-cells in CP patients secreted high levels of IFN-γ and low levels of IL-4 (a cytokine secreted by Th2 cells) following stimulation with lipopolysaccharide, indicating polarisation towards the Th1 phenotype. However it must be noted that the authors did not specifically identify CD4+ T-cells as the source of IFN-γ, a cytokine that can also be produced by CD8+ cytotoxic T-cells.[136] Two other studies analysed peripheral blood Treg cells in CP however their results were discordant: One author noted that Treg cells were significantly expanded in the blood of CP patients compared to controls;[139] but another found no difference existed.[140]

In other inflammatory diseases there is frequently a polarisation of T-helper lymphocyte responses towards either a Th1 or Th2 phenotype. My data clearly demonstrates that Th1 cells are found in significantly higher numbers in CP patients compared to controls, and they are a prominent source of IFN-γ. An intriguing observation is the effect that excess alcohol consumption has on the proportion of Th1 cells observed. As chronic pancreatitis is most frequently related to excessive alcohol consumption, which may lead to increased gut permeability and high circulating levels of LPS [169, 170] it is tempting to speculate that this promoted the increased numbers of Th1 cells seen when compared to non-drinkers. LPS is well-known for generating Th1 cell responses thus this may be important in the pathogenesis of the disease.[171] Excess alcohol consumption had no effect on other T-helper cell subsets, nor did smoking. Given that only a minority of heavy drinkers develop chronic pancreatitis,[25] this may represent one of the factors that govern the pathophysiology of the disease. This merits further examination, however it should be noted that the number of drinkers was small in the groups examined which may have biased the findings.

In addition to the Th1 findings this work is the first to demonstrate a significant increase in the number of IL-17 and IFN-γ/IL-17 secreting Th17 cells in the peripheral blood of CP patients compared to controls. It also has unusually found that there is no polarisation of the T-helper cell response in the peripheral blood of CP patients towards either a Th1 or Th2 phenotype. As Th2 and Treg cells are increased in CP, along with Th1 and Th17 cells, it appears that all T-cell lineages are specifically primed to respond

to inflammatory stimuli, although interestingly there was no difference in the proportion of activated $CD25^+$ T-cells in CP patients or controls.

Th1 cells act to stimulate phagocyte-mediated defence against infections by activating macrophages and promoting opsonisation, whereas Th17 cells appear to play a critical function in protection against extracellular bacteria. They are thus both designed to orchestrate an inflammatory response orientated towards pathogen clearance by recruiting and activating inflammatory leukocytes. They can however also cause bystander tissue damage and elicit unwanted inflammatory disease and self-reactivity. Th2 cells on the other hand are not only important in controlling mucosal immunity but they also produce anti-inflammatory cytokines such as IL-4 and IL-13 that antagonise the activation of macrophages stimulated by IFN-γ. Th2 cells therefore are not just effector Th-cells but also regulators of the immune response, whereby the late appearance of Th2 cells may limit the injurious consequences of Th1 cell mediated immunity. Furthermore Th2 responses promote collagen deposition and fibrosis, whilst Th1 and Th17 responses inhibit it by causing extra-cellular matrix degradation, indicating opposing actions in tissue repair. In addition Treg cells play an important function in maintaining immune homeostasis and suppressing harmful immunopathological responses to self or foreign antigen. The reciprocal production of iTreg and Th17 cells from naïve $CD4^+$ T-cells provides supplementary and allied means of balancing immune responses.

Infectious and inflammatory diseases frequently demonstrate a bimodal immune reaction. Initially Th1 and Th17 cells react to invading pathogens or chronic irritants, however if the immune stimulus persists, Th2 and Treg mediated suppressive mechanisms are activated to prevent excessive damage to host tissues and promote wound healing.[103] It is likely therefore that the changes seen in the peripheral blood of CP patients reflects a continuous equilibrium of destructive inflammation and reparative fibrosis, which ultimately results in loss of functioning exocrine and endocrine tissue, and the characteristic symptoms of chronic pancreatitis. Higher numbers of Th1, Th2, Th17 and Treg cells are therefore found in the peripheral blood of CP patients which reflects the ongoing underlying inflammatory process present within the pancreas.

T-HELPER AND T-REGULATORY LYMPHOCYTES IN PANCREATIC TISSUE

Pancreatic tissue from four patients with chronic pancreatitis (all male, median age 41 years, age range 35-47 years) (See Table 13: P04, P13, P23, P34) was enzymatically and mechanically digested to allow the isolation of mononuclear cells, which were cryopreserved and subsequently thawed, stimulated and analysed using flow cytometry to detect T-helper cells as previously described. The isolated pancreatic mononuclear cells from patients P23 and P34 were also analysed for T-regulatory cells. As normal pancreatic tissue was shown to contain few T-cells when analysed using flow cytometry it was not assessed for T-helper or T-regulatory cell lineages. PBMC analysed at the same time served as positive controls.

In addition, GMA embedded pancreatic tissue was assessed using immunohistochemical methods as previously described. The tissue of six patients with chronic pancreatitis (5 male, 1 female, median age 54 years, age range 35-75 years) (See Table 13: P04, P13, P19, P29, P44, P46) and four control patients (1 male, 3 female, median age 63 years, age range 47 – 75 years) (See Table 14: P07, P10, P12, P45) was assessed.

Chronic pancreatitis tissue analysed using flow cytometry demonstrated that the T-helper cell infiltrate was predominantly comprised of IFN-γ^+ Th1 cells (median 41.5%) (See Figure 26 and Figure 27). Interestingly the lowest number of Th1 cells was seen in patient P23, a patient with idiopathic CP (See Figure 27). IL-17^+ Th17 cells were also seen (median 2.1%), including dual secreting IFN-γ^+ IL-17^+ Th17 cells (median 0.37%) (See Figure 26 and Figure 27). In contrast, no discernible IL-13^+ Th2 cells were identified (See Figure 28) and few or no Treg cells were seen (See Figure 29).

When pancreatic tissue was analysed using immunohistochemistry the number of IFN-γ^+ cells was significantly higher in the tissue of patients with chronic pancreatitis than controls (CP median 0.84 cells/mm^2 *vs.* 0 cells/mm^2; p=0.023) (See Figure 30 and Figure 31). The cellular source of the IFN-γ is unclear however as it may be secreted by a number of cells including T-lymphocytes and macrophages. The number of IFN-γ^+ cells sometimes exceeded the number of $CD4^+$ T-cells confirming that T-helper cells are not its only source (See Table 24).

No positive cellular staining for IL-13, IL-17 or FoxP3 was seen in chronic pancreatitis or normal control pancreatic tissue using immunohistochemistry. Positive control tonsil

tissue mounted on the same slide as each piece of pancreatic tissue confirmed that the immunohistochemical staining process had worked (See Figure 32 and Figure 33).

Figure 26: Dot Plots Showing the Proportion of Th1 and Th17 Cells in Chronic Pancreatitis Tissue

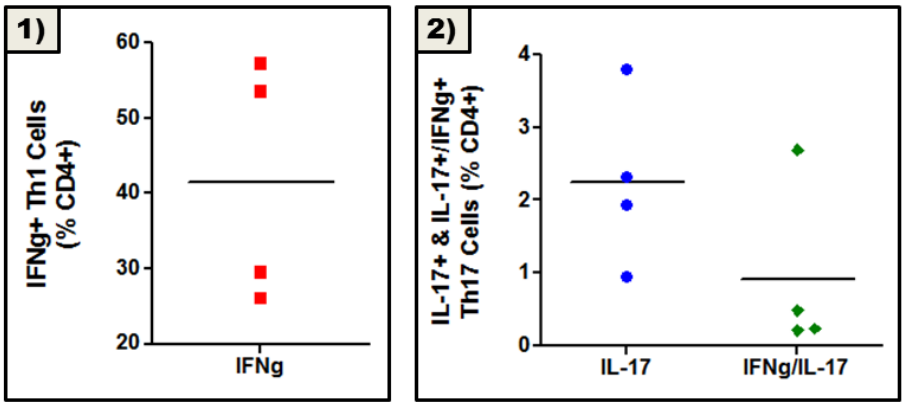


Figure 26 Legend

The pancreatic tissue of four patients with chronic pancreatitis was analysed using flow cytometry to identify CD4+ T-helper cells: 1) IFN-γ^+ Th1 cells; 2) IL-17^+ and dual secreting IFN-γ^+IL-17^+ Th17 cells. The black line shows the median for each data set.

Figure 27: Flow Cytometry Results of Chronic Pancreatitis Tissue Th1 and Th17 Cells

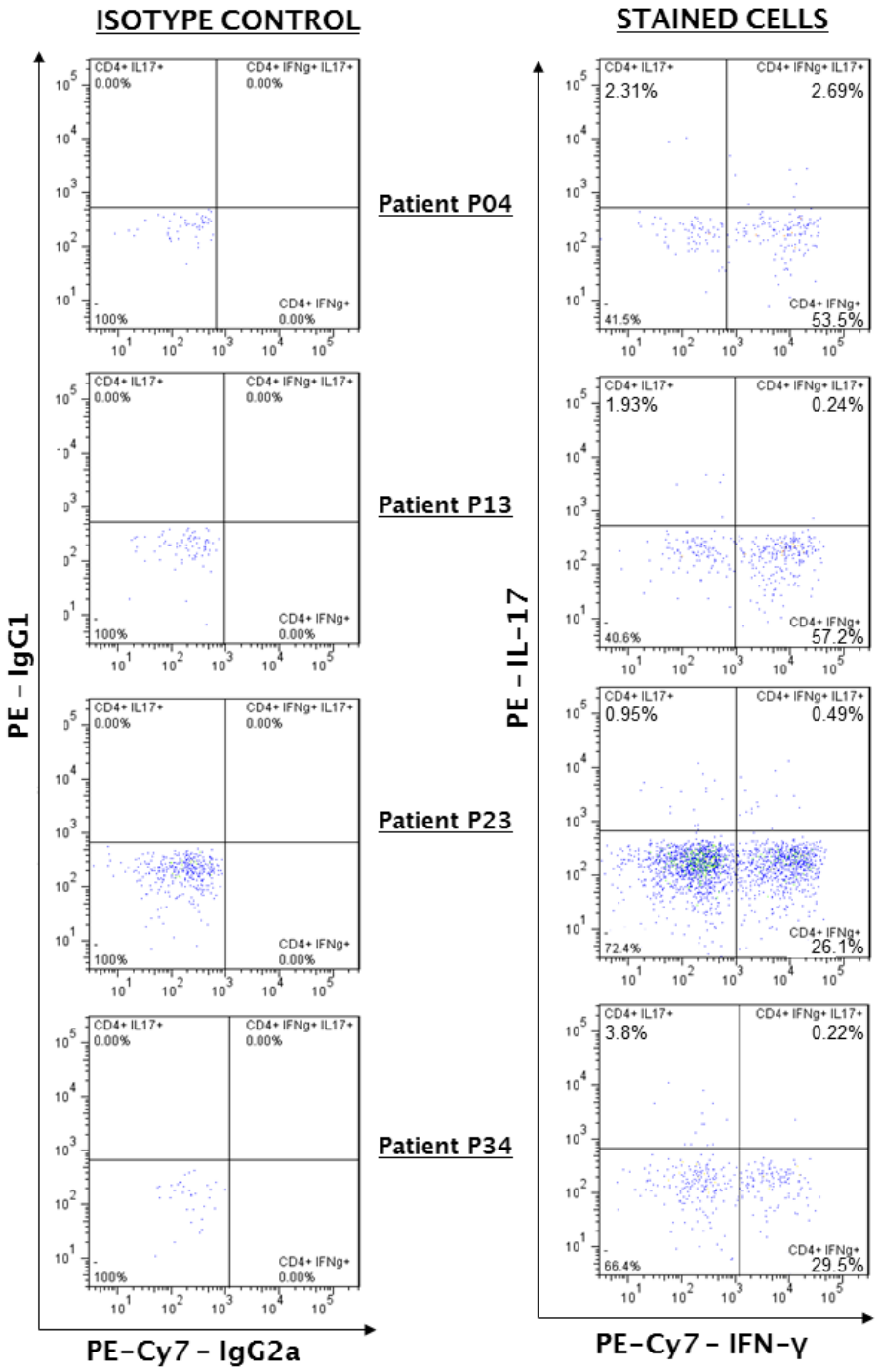


Figure 27 Legend

Lymphocytes were extracted from the pancreatic tissue of four patients with chronic pancreatitis (See Table 13), stimulated with PMA and ionomycin, and then stained with either isotype control antibodies or for intracellular cytokines. Results of all patients are shown, gated on CD4+ T-cells. Significant numbers of Th1 and Th17 cells were identified, including a small but noteworthy number of dual secreting IFN-γ^+IL-17^+ Th17 cells.

Figure 28: Flow Cytometry Dot Plots of Chronic Pancreatitis Tissue Th1 and Th2 Cells

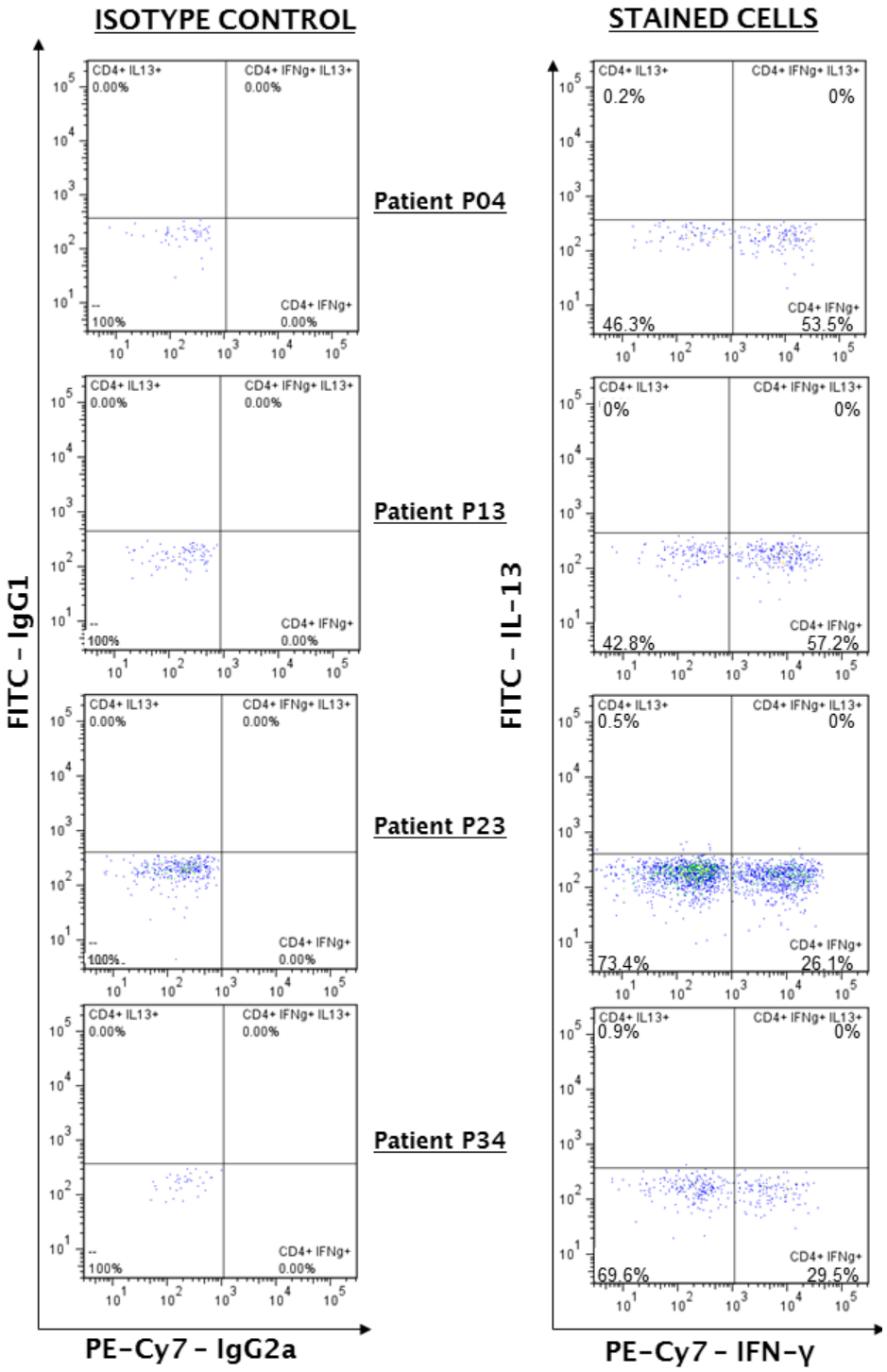


Figure 28 Legend

Lymphocytes were extracted from the pancreatic tissue of four patients with chronic pancreatitis (See Table 13), stimulated with PMA and ionomycin, and then stained for either isotype control antibodies or for intracellular cytokines. Results of all patients are shown, gated on CD4+ T-cells. Significant numbers of Th1 cells were identified but no discernible Th2 cell population was seen.

Figure 29: Flow Cytometry Results for Pancreatic Tissue T-Regulatory Cells

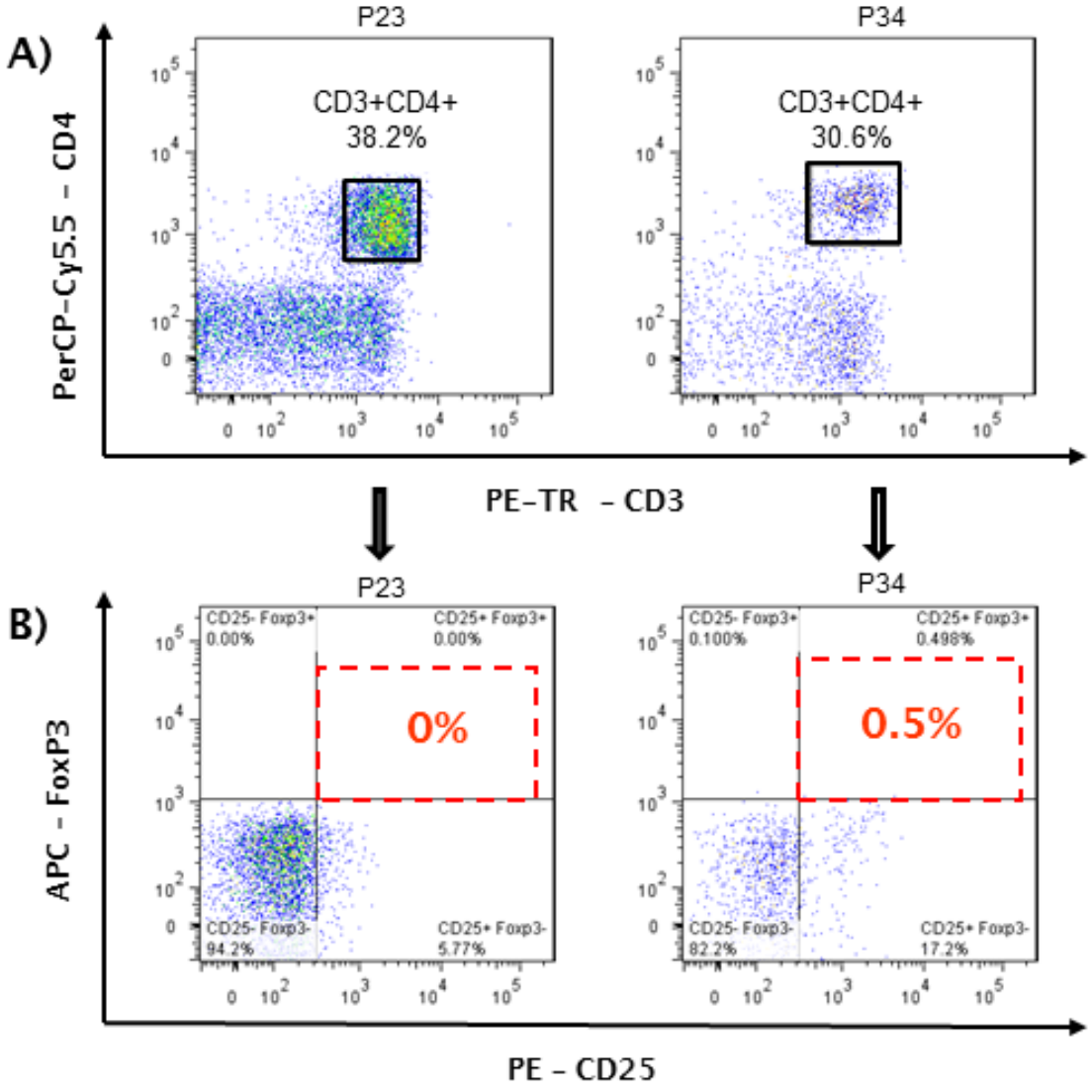


Figure 29 Legend

Lymphocytes were extracted from pancreatic tissue of two patients with chronic pancreatitis (See Table 13: P23 and P34) and analysed using flow cytometry to identify T-regulatory (Treg) lymphocytes. A) Good yields of $CD3^{+}CD4^{+}$ T-cells were obtained from each patient (gated on live singlet tissue lymphocytes); B) Few or no $CD25^{+}FoxP3^{+}$ Treg cells were identified (gated on $CD3^{+}CD4^{+}$ T-cells). Positive control PBMC stained at the same time demonstrated positive staining (not shown).

Figure 30: Dot Plots of Cells Expressing IFN-γ and CD4 in Pancreatic Tissue Identified Using Immunohistochemistry

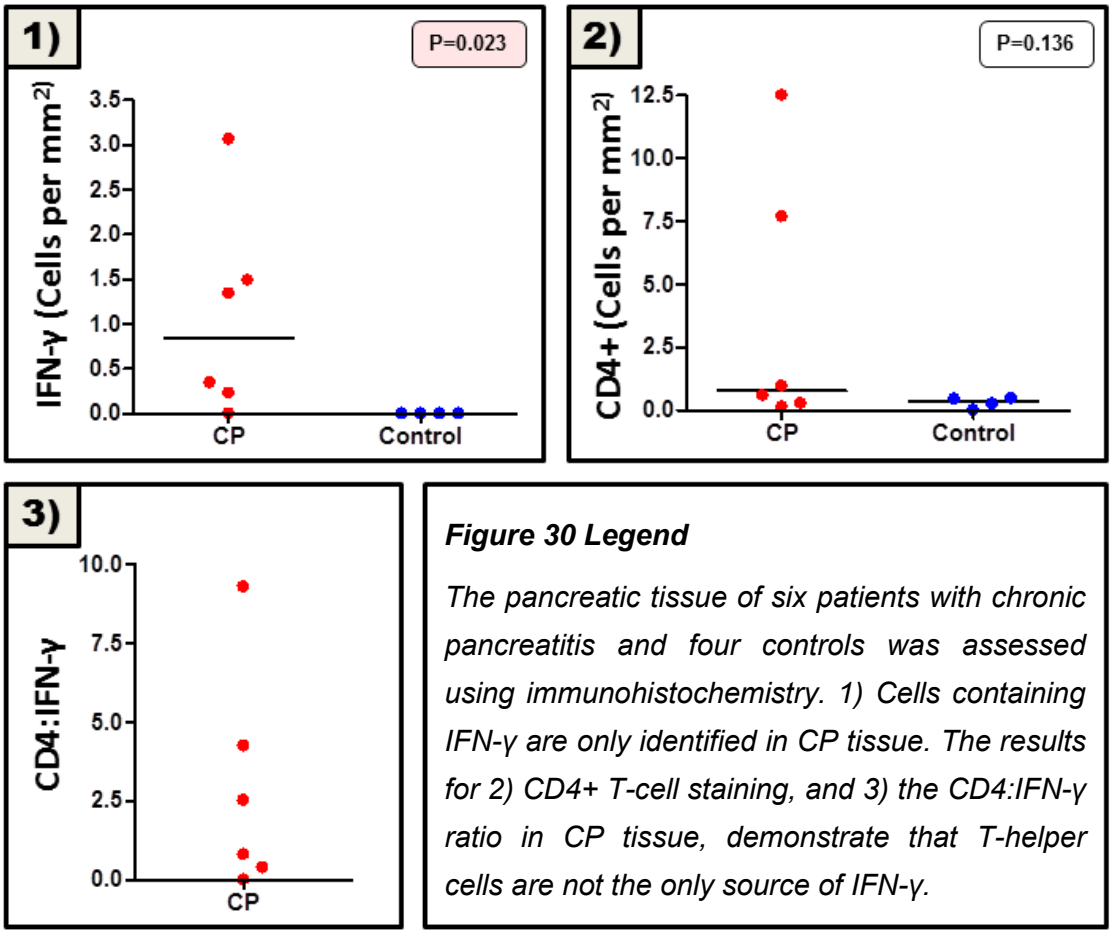


Figure 30 Legend

The pancreatic tissue of six patients with chronic pancreatitis and four controls was assessed using immunohistochemistry. 1) Cells containing IFN-γ are only identified in CP tissue. The results for 2) CD4+ T-cell staining, and 3) the CD4:IFN-γ ratio in CP tissue, demonstrate that T-helper cells are not the only source of IFN-γ.

Table 24: CD4 and IFN-γ Immunohistochemistry Results in Chronic Pancreatitis

Patient ID	CD4 (cells/mm^2)	IFN-γ (cells/mm^2)	CD4:IFN-γ
P04	7.69	3.06	2.52
P13	0.13	0	0
P19	0.28	0.35	0.80
P29	0.59	1.49	0.39
P44	0.96	0.23	4.52
P46	12.50	1.34	9.30

Table 24 Legend

The data from immunohistochemical analysis of chronic pancreatitis tissue. Patients may be identified from Table 13.

Figure 31: IFN-γ Positive Cells Identified in Human Tonsil and Chronic Pancreatitis Tissue Using Immunohistochemistry

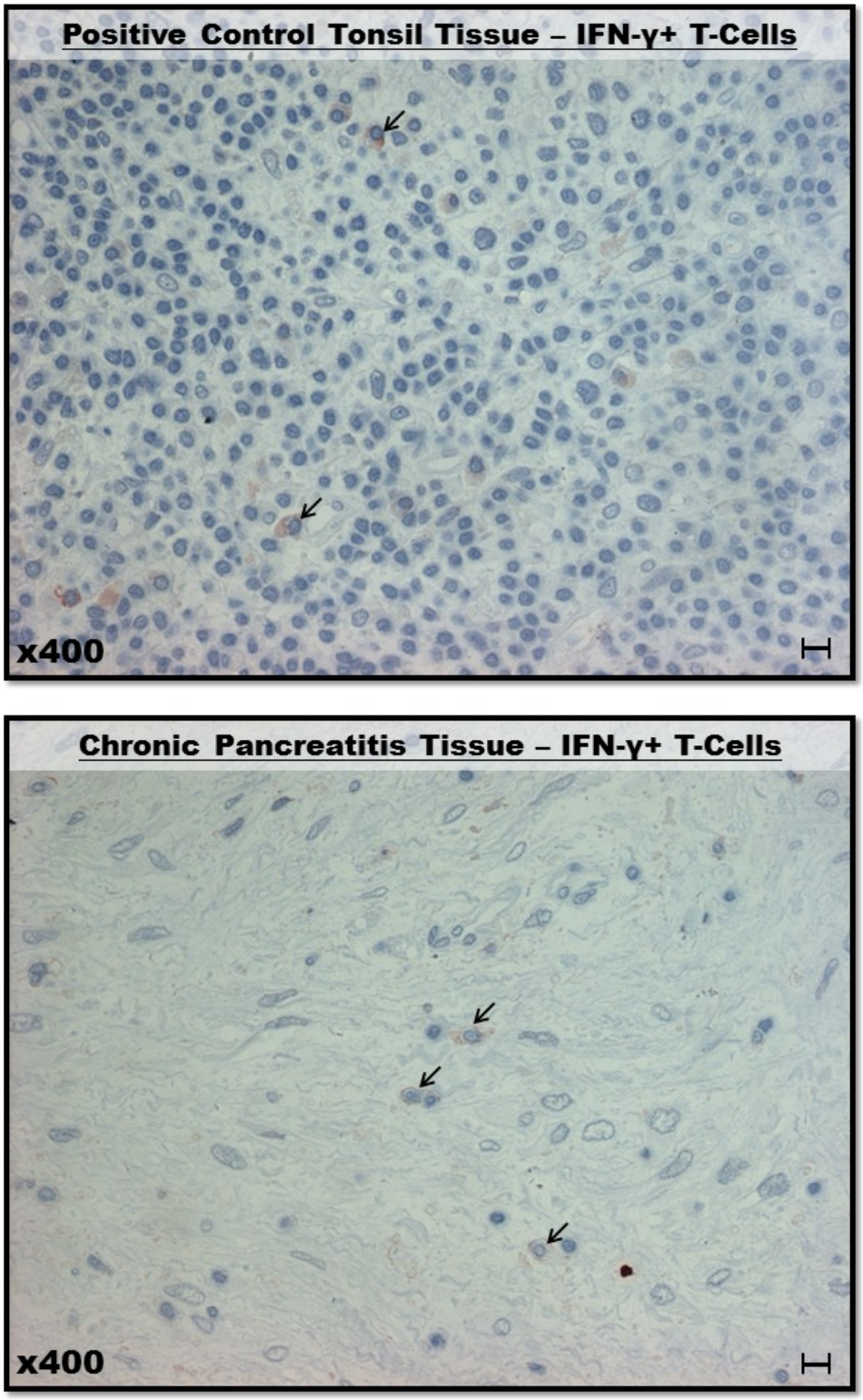


Figure 31 ***Legend***

IFN-γ^+ cells (arrowed) are identified by red cytoplasmic staining in GMA embedded human positive control tonsil tissue (upper image) and chronic pancreatitis tissue (lower image).

Figure 32: Identification of IL-13 and IL-17 Positive Cells in Human Tonsil Using Immunohistochemistry

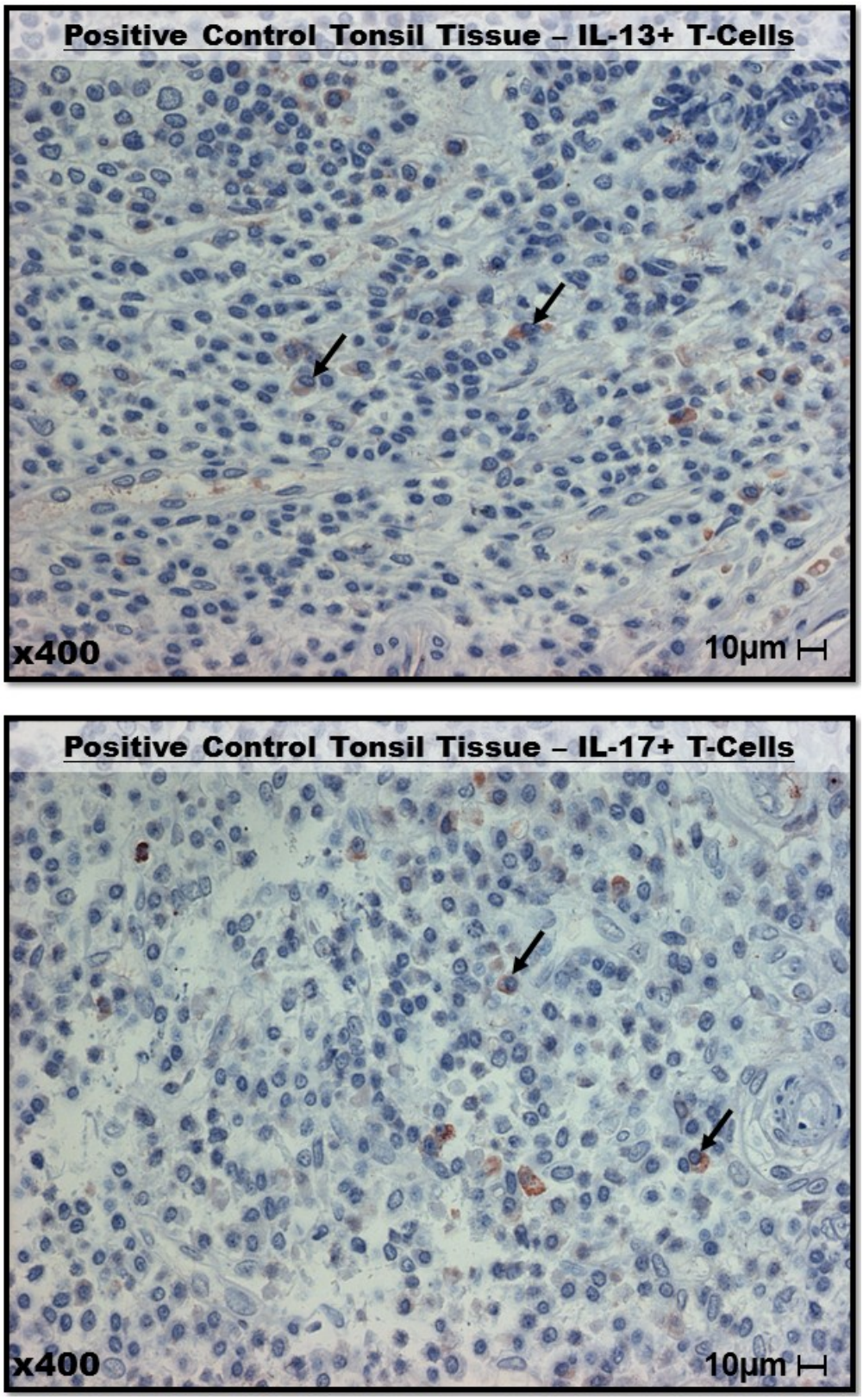


Figure 32 ***Legend***

IL-13+ (upper image) and IL-17+ (lower image) T-cells (arrowed in each) are identified by red cytoplasmic staining in GMA embedded human positive control tonsil tissue.

Figure 33: Positive Control Human Tonsil FoxP3 Immunohistochemical Staining

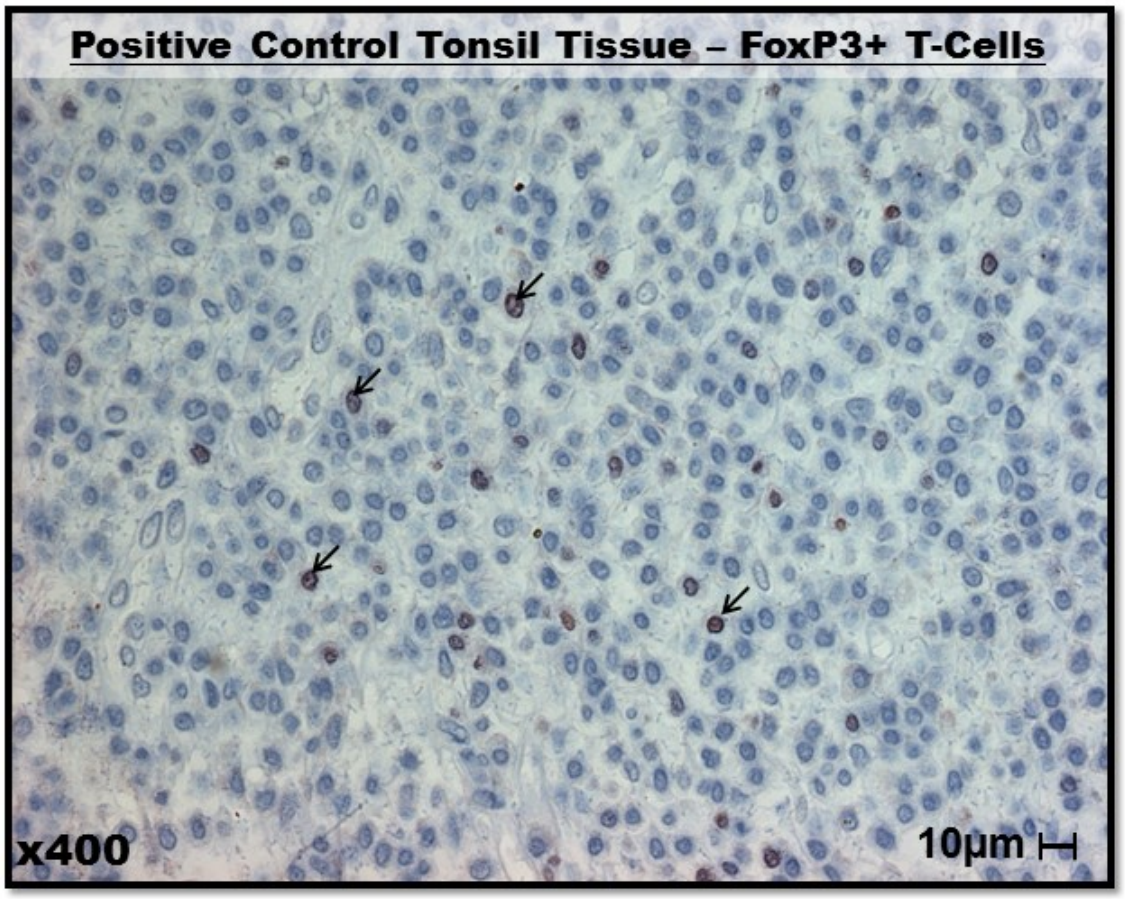


Figure 33 Legend

GMA embedded human positive control tonsil tissue. FoxP3+ T-cells (arrowed) are identified by red nuclear staining.

Conclusion

The results from the flow cytometry examination of chronic pancreatitis tissue clearly demonstrate that the T-helper cell infiltrate is predominantly comprised of IFN-γ secreting Th1 cells. In addition Th17 cells, some of which secrete both IL-17 and IFN-γ, were also detectable in appreciable numbers, however IL-13 secreting Th2 cells were not detected. IL-18, a pro-inflammatory cytokine that promotes IFN-γ production in T-cells, has previously been found to have significantly higher expression in CP tissue compared to controls supporting these findings.[218] Intriguingly the patient with idiopathic CP (P23), rather than alcoholic CP (all others), had the lowest number of Th1 cells. This further supports the notion that alcohol alters T-helper cell homeostasis promoting a Th1 predominant phenotype.

A significantly higher number of positively stained IFN-γ containing cells were seen in inflamed chronic pancreatitis tissue compared to controls when assessed using immunohistochemistry. Although the specific cellular localisation of IFN-γ was not identified it is likely that a substantial proportion of the IFN-γ^+ cells were T-helper 1 lymphocytes. It is possible to use co-localisation techniques or dual staining with different chromagens to formally establish the cellular localisation of cytokines, however the flow cytometry results are convincing enough evidence to support the immunohistochemistry findings in this case.

It is interesting that IL-17+ cells were not detected using immunohistochemistry. I think this is likely explained by the fact that T-cells do not store cytokines. When they are fixed in tissue however a limited quantity of cytokine is present within their cytoplasm which may then be identified. Th17 cells were few in number when pancreatic tissue was analysed using flow cytometry. The fact that no IL-17 containing T-cells were identified using immunohistochemistry may therefore reflect that either the sensitivity to detect them is too low or that the amount of tissue assessed was too small and did not allow a reasonable chance to identify them. This latter point is explained by the fact that approximately fifty CD4+ T-cells would need to be identified to have a good chance of seeing one Th17 cell, as the median percentage of CD4+ Th17 cells identified using flow cytometry was 2.12%. The chronic pancreatitis tissue assessed using immunohistochemistry contained 0.77 CD4+ T-cells per mm^2 and the median area of tissue examined was 5.55mm^2. It is unlikely therefore that a Th17 cell would have been clearly identified.

The failure to identify Treg cells in chronic pancreatitis tissue using either flow cytometry or immunohistochemistry conflicts with the work by previous authors, in particular Schmitz-Winnenthal *et al* who found that Treg cells were significantly enriched in CP tissue compared to peripheral blood.[139] It may well be that the patients analysed by this group were different from mine, and had more advanced disease or an inflammatory mass. It is unlikely that an inflammatory process would exist that did not have an element of inhibitory control, so as for IL-17, my failure to detect Treg cells using immunohistochemistry may have been caused by the numbers being below the lower limit of sensitivity for the method. The failure to detect Treg cells using flow cytometry is more difficult to explain as positive control PBMC stained well. As the results are the product of only two experiments it is possible there were technical errors in each process. Resolution of these aberrant findings could be undertaken in the

future by increasing the amount of tissue analysed by immunohistochemistry and repeating the flow cytometry experiments.

MUCOSAL-ASSOCIATED INVARIANT T-LYMPHOCYTES IN PERIPHERAL BLOOD

PBMC isolated from chronic pancreatitis patients and controls were analysed using flow cytometry as previously described. The blood of 23 patients with CP and 16 controls was assessed (See Table 25). Mucosal-associated invariant T (MAIT) – cells were identified as $CD3^+$ $CD4^-CD8^-/CD4^-CD8^+$ $CD161^+$ TCR $V\alpha7.2^+$ (See Figure 34). Statistical analysis was undertaken using a Mann-Whitney U Test.

Table 25: Patient Demographics of Peripheral Blood MAIT Cells Analysed Using Flow Cytometry

	Chronic Pancreatitis		Control	
Total Number	23		16	
Gender	21 Male / 2 Female		12 Male / 4 Female	
Median Age (years)	47.0	47.0 M 62.5 F	50.00	50.0 M 54.5 F
Age Range (years)	25 – 74	25 – 74 M 61 – 64 F	32 - 75	32 – 73 M 42 – 75 F

Table 25 Legend

The specific demographic details for each chronic pancreatitis patient (P04, P08, P09, P11, P13, P14, P15, P16, P18, P19, P23, P24, P25, P28, P29, P34, P35, P36, P37, P39, P42, P44, P46) and control patient analysed (P07, P12, P26, P33, P40, P41, P43, P45, P47, P48, P49, P50, P51, P52, P54, P55) can be seen in Table 13 and Table 14 respectively.

No significant difference between all MAIT, CD8+ MAIT and DN MAIT cells was observed between CP patients and controls cells (all MAIT: 6.23% *vs.* 10.07%; p= 0.32. CD8+ MAIT: 2.11% *vs.* 4.96%; p=0.13. DN MAIT: 3.31% *vs.* 4.63%; p=0.36) (See Table 26 and Figure 35). There does however appear to be a subtle trend towards a reduction in the proportion of CD8+ MAIT in the peripheral blood of CP patients compared to controls.

It should be noted that the results of the analysis of CD4+ and CD8+ T-cell subsets in this patient cohort varies slightly from that analysed previously (See page 100). In both patient cohorts a trend towards a decrease in the proportion of CD4+ T-cells was

observed. However in this patient cohort a trend towards an increase in the proportion of CD8+ T-cells was also seen, which was not present in the other patient cohort. The results of both statistical analyses can be seen in Table 26 and an extract of Table 16.

Figure 34: Identification of Mucosal-Associated Invariant T-Cells in Peripheral Blood

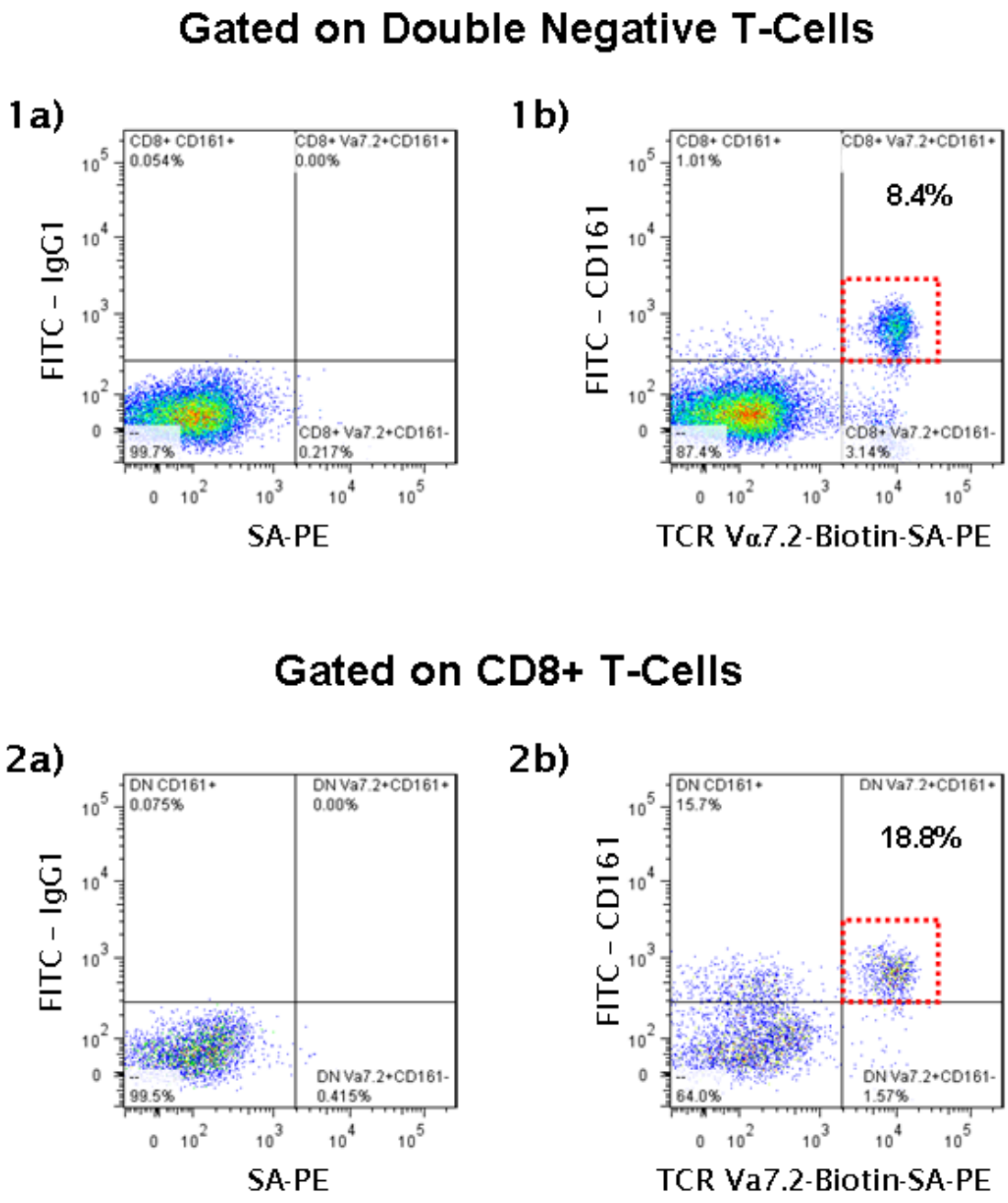


Figure 34 Legend

PBMC were isolated from a healthy blood donor. Double negative (DN) and CD8+ T-cells were identified using the gating strategy shown in Figure 12. A negative gate was created using matched isotype control antibodies and streptavidin(SA)-PE (1a) and (2a). DN (1b) and CD8+ (2b) mucosal-associated invariant T-cells (MAIT) were identified using a FITC labelled anti-CD161 antibody and a biotinylated anti-T-cell receptor (TCR) Vα7.2 antibody which was detected with SA-PE. The same strategy was employed to identify pancreatic tissue infiltrating MAIT cells (See Figure 36).

Figure 35: Dot Plots Showing the Results of Peripheral Blood MAIT Cell Analysis in Chronic Pancreatitis Patients and Controls Using Flow Cytometry

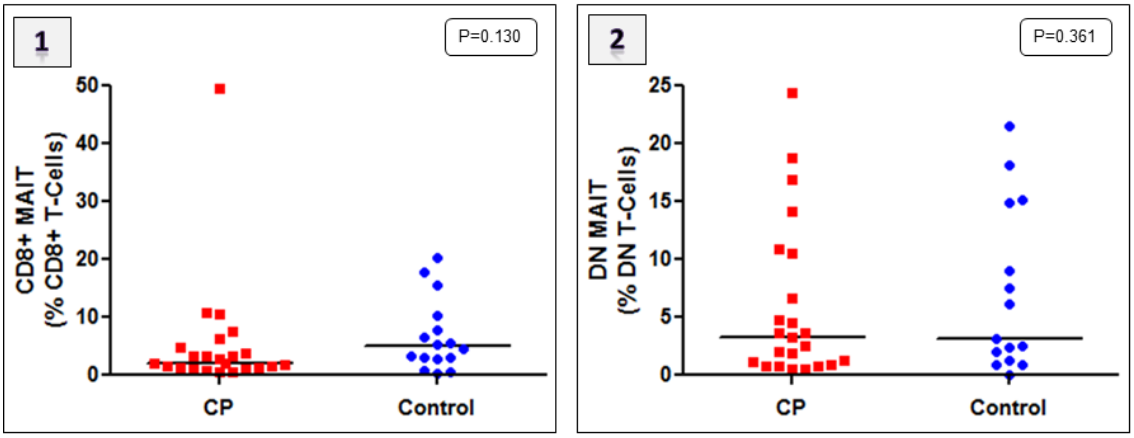


Figure 35 Legend

1) CD8+ MAIT cells and 2) double negative (DN) MAIT cells were identified in the peripheral blood of patients with chronic pancreatitis and controls. Black lines show the median for each data set. An outlier in the CP CD8+ MAIT group, a patient with idiopathic CP (See Table 13: P23), can be clearly seen.

Table 26: Results of Flow cytometry Analysis of Peripheral Blood MAIT Cells

T-helper Lymphocyte Lineage	Chronic Pancreatitis		Control		P value
	Median	Interquartile Range	Median	Interquartile Range	
CD4+ (% of CD3+)	64.50	55.20 - 72.40	71.95	62.25 - 80.70	**0.087**
CD8+ (% of CD3+)	25.00	19.8 - 33.3	19.70	14.05 - 26.50	**0.079**
DN (% of CD3+)	5.87	3.63 - 8.2	5.29	3.66 - 7.88	0.775
CD4:CD8	2.54	1.69 - 3.81	3.63	2.38 - 5.49	**0.082**
All MAIT (% of CD3+)	6.23	2.45 – 15.41	10.07	4.70 – 22.42	0.318
CD8+ MAIT (% of CD8+)	2.11	1.17 - 4.81	4.96	2.90 - 9.59	0.130
DN MAIT (% of DN)	3.31	0.87 - 10.50	4.63	1.46 - 15.13	0.361

Extract of Table 16: Results of Peripheral Blood T-Lymphocyte Subset Analysis Using Flow Cytometry

T- Lymphocyte	Chronic Pancreatitis		Control		P value
	Median	Interquartile Range	Median	Interquartile Range	
CD4+ (% of CD3+)	66.65	56.58 - 71.18	73.10	60.30 - 81.23	**0.076**
CD8+ (% of CD3+)	23.95	19.48 - 32.53	20.85	12.30 - 29.30	0.130
DN (% of CD3+)	4.96	2.73 - 8.27	4.30	2.30 - 6.33	0.288
CD4 to CD8 ratio	2.80	1.72 - 3.65	3.53	2.06 - 6.60	**0.097**

Conclusion

This is the first study to assess peripheral blood MAIT cells in the context of CP. A trend towards a lower proportion of CD8+ MAIT cells exists in the peripheral blood of chronic pancreatitis patients compared to controls. However the proportion of DN MAIT cells is similar in both disease and control groups. Previous authors have demonstrated that infective and inflammatory processes result in fewer MAIT cells in peripheral blood. In particular fewer MAIT cells are found in the blood of patients with pulmonary *Mycobacterium tuberculosis* infection compared to healthy controls, and instead MAIT are enriched in infected lung lesions.[112] The frequency of MAIT cells in peripheral blood of multiple sclerosis patients is also significantly reduced compared to controls, with this difference being more profoundly evident in patients experiencing a relapse in their disease.[113] My data therefore suggests that MAIT cells are likely to be involved in non-specific inflammation. The further analysis of MAIT cells in pancreatic tissue follows this section.

The differences observed between the CD4+ and CD8+ T-cell populations in the two patient cohorts described demonstrates that the size of the data sets may lead to both type 1 error (falsely rejecting the null hypothesis) and type 2 error (falsely accepting the null hypothesis). Increasing the sample size is the most legitimate way of removing the likelihood of error, however this clearly would require extension of this study. Visual interpretation of graphical data in Figure 15 suggests that the trends demonstrated are most likely true.

MUCOSAL-ASSOCIATED INVARIANT T-LYMPHOCYTES IN PANCREATIC TISSUE AND PERIPHERAL BLOOD

Mononuclear cells from eight CP patients (all male, median age 46.5 years, age range 35-63 years) (See Table 13: P04, P13, P19, P23, P29, P34, P44, P46) were analysed using flow cytometry as previously described. MAIT cells were identified as shown in Figure 34. Statistical analysis was performed using the Wilcoxon Signed Ranks Test. It should be noted that the pancreatic tissue of control patients contained few or no T-cells and no MAIT cells were concordantly identified. Normal pancreatic tissue is therefore not included in this analysis.

Whilst no difference was observed in the total number of MAIT cells found in the pancreatic tissue and peripheral blood of patients with CP (17.74% *vs.* 12.52%; p=0.26), a significant enrichment of double negative (DN) MAIT cells was seen when pancreatic tissue was compared to peripheral blood (9.08% *vs.* 4.09%; p=0.017) (See Table 27, Figure 36 and Figure 37). However no difference existed between CD8+ MAIT cells in either body compartment (4.20% *vs.* 4.77%) (See Table 27).

Table 27: Paired Pancreatic Tissue and Peripheral Blood MAIT Cell Analysis Using Flow Cytometry

Lymphocyte Subset	Blood		Tissue		P value
	Median	Interquartile Range	Median	Interquartile Range	
All MAIT (% of CD3+)	12.52	2.58 – 24.20	17.74	7.10 – 35.53	0.263
CD8+ MAIT (% of CD8+)	4.77	0.79 - 10.09	4.21	0.77 - 10.49	0.327
DN MAIT (% of DN)	4.09	1.70 - 11.84	9.08	5.87 - 13.2	**0.017**

Figure 36: Paired Results of Chronic Pancreatitis Tissue and Peripheral Blood Analysis Showing DN MAIT Cells Identified Using Flow Cytometry

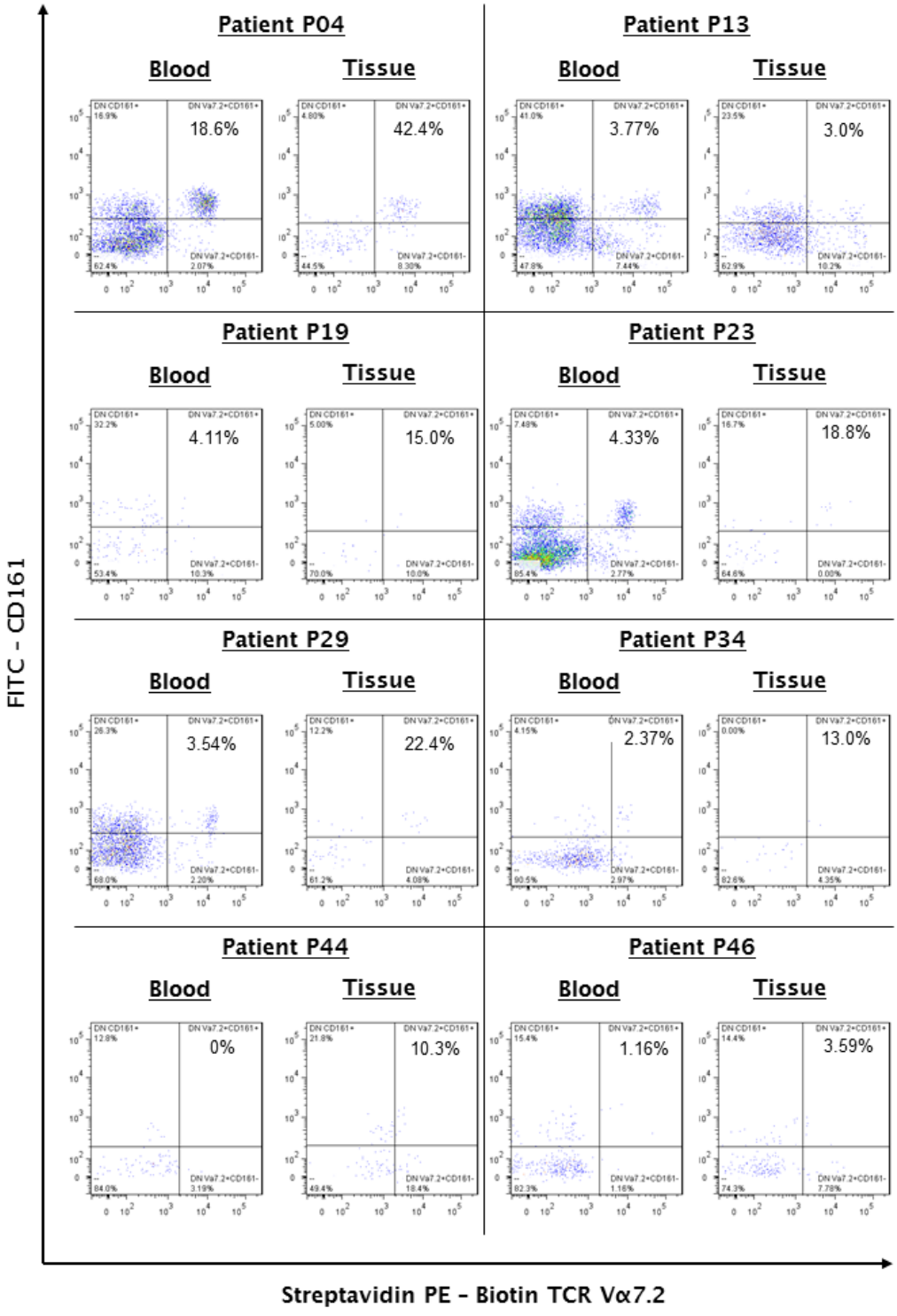


Figure 36 Legend

The pancreatic tissue and peripheral blood from eight patients with chronic pancreatitis was analysed using flow cytometry to identify double negative mucosal-associated invariant T-cells. The dot plots for each patient (See Table 13) are shown.

Figure 37: Dot Plot Showing Paired Results of Chronic Pancreatitis Tissue and Peripheral Blood Double Negative MAIT Cells

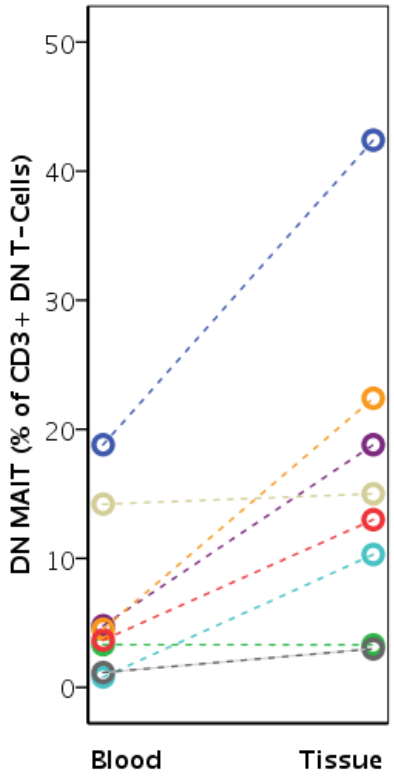


Figure 37 Legend

The peripheral blood and pancreatic tissue of eight patients with CP was analysed using flow cytometry. The proportion of DN MAIT cells as a percentage of total CD3+ DN T-cells is shown for peripheral blood (median 4.09%) and pancreatic tissue (median 9.08%) in each patient as paired data points. A significant enrichment of DN MAIT cells is seen in the pancreatic tissue of CP patients (p=0.017).

Conclusion

This study is the first to identify MAIT cells in CP tissue. Furthermore the finding that double negative MAIT cells are enriched in the inflamed pancreatic tissue of patients with chronic pancreatitis demonstrates a particularly interesting finding. MAIT play a role in the identification and elimination of microbial pathogens, in particular *Escherichia coli* and *Staphylococcus aureus*,[111] both of which may cause pathogenic intestinal infections. They have also been found to become enriched in the lung tissue of patients infected with *Mycobacterium tuberculosis*.[112] My data therefore suggest that MAIT cells are recruited not only to a site of inflammation but possibly also pancreatic infection. Bacteria may have entered pancreatic tissue via a number of potential means, most likely by translocation across the intestinal lumen. As described earlier alcohol causes the intestinal lumen to become increasingly permeable promoting bacterial entry into the circulation.[177] Furthermore small bowel bacterial overgrowth is common in CP patients.[178] As pancreatic tissue is normally free of pathogenic microbes it is possible that DN MAIT have entered chronically inflamed pancreatic tissue to eliminate them.

MAIT may also have a role in regulating and suppressing tissue inflammation. MAIT isolated from patients with multiple sclerosis have been shown *in vitro* to suppress IFN-γ production by Th1 cells.[113] I have shown that Th1 cells are the predominant T-helper lymphocyte found in CP tissue and that there are significantly higher numbers of Th1 cells in the peripheral blood of CP patients compared to controls. These novel findings may therefore be connected and in the future should be examined in more detail to establish what functional roles they play and if there is functional cross-talk between these two lymphocyte lineages.

IDENTIFICATION OF THE VA7.2 AND JA33 INVARIANT T-CELL RECEPTOR RE-ARRANGEMENT FOLLOWING FLUORESCENCE ACTIVATED CELL SORTING OF MUCOSAL-ASSOCIATED INVARIANT T-CELLS

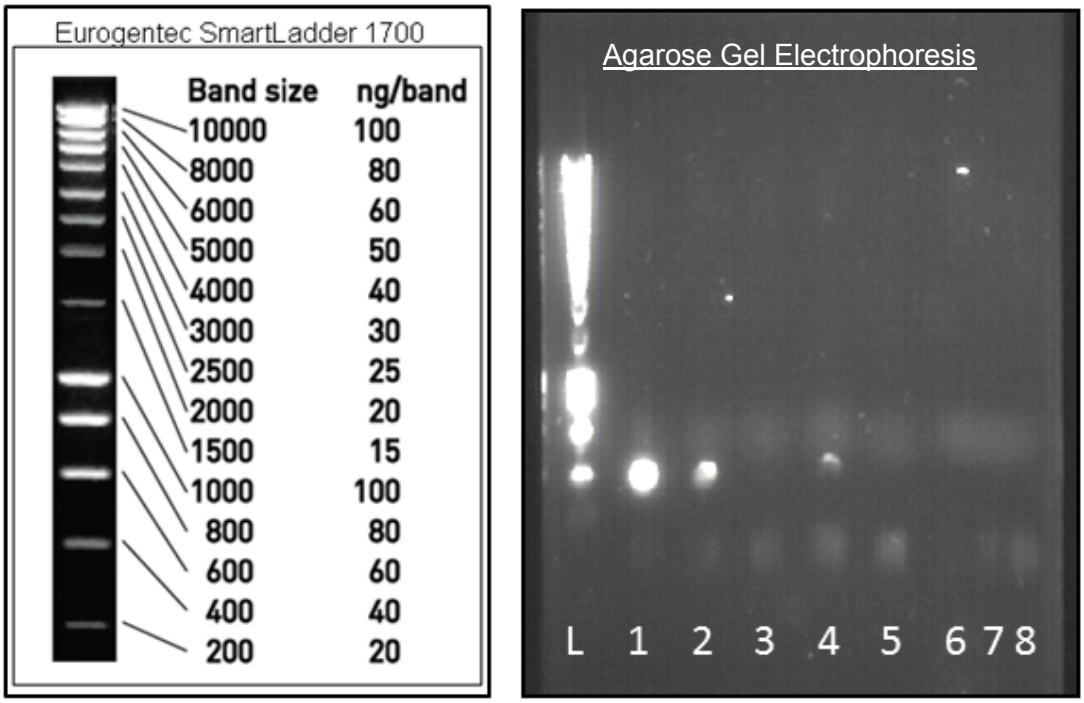
PBMC from a healthy donor (See Table 14: P55) were isolated and then stained with fluorescent antibodies and dyes. Fluorescence activated cell sorting was then utilised to isolate live $CD3^+$ TCR $V\alpha7.2^+$, $CD3^+$ TCR $V\alpha7.2^-$, $CD3^+$ $CD4^-CD8^+$ $CD161^{+/-}$ TCR $V\alpha7.2^+$ and $CD3^+$ DN $CD161^{+/-}$ TCR $V\alpha7.2^+$ T-cells. RNA was subsequently extracted from the sorted T-cells which was reverse transcribed to make cDNA before PCR was performed using primers to amplify the invariant Vα7.2-Jα33 TCR re-arrangement characteristic of MAIT cells. Agarose gel electrophoresis on the PCR products was then finally used to identify the presence or absence of MAIT cells (See Figure 38). Unfortunately analysis of MAIT cells isolated from two patients with CP was spoiled by contamination of the PCR product by an unidentified source (not shown).

The Vα7.2-Jα33 TCR re-arrangement was found in DN $CD161^+$ TCR $V\alpha7.2^+$ and $CD8^+$ $CD161^+$ TCR $V\alpha7.2^+$ T-cells, and not in either the DN or $CD8^+$ $CD161^-$ TCR $V\alpha7.2^+$ T-cells populations. It should be noted that the numbers of cells analysed was small in the latter two populations but within the likely range of detection.

Conclusion

It is clear that true MAIT cells are $CD161^+$ and that the antibody employed to identify the Vα7.2 TCR was appropriately specific. It is a great shame that contamination of the PCR product from MAIT cells isolated from CP patients occurred, particularly as the source of the contamination could not be identified.

Figure 38: PCR and Agarose Gel Electrophoresis of Peripheral Blood MAIT Cells



Primers = TCR Vα7.2 Forward & TCR Jα33 Reverse	Band Size = 376

L.	Eurogentec SmartLadder 1700
1.	$CD3^+$ T-Cell Positive Control cDNA
2.	DN TCR-Vα7.2^+ $CD161^+$ (sorted 917 cells; used 1/15 of cDNA)
3.	DN TCR-Vα 7.2^+ 161^- (sorted 86 cells; used 1/15 of cDNA)
4.	CD8+ TCR-Vα 7.2^+ $CD161^+$ (sorted 1343 cells; used 1/15 of cDNA)
5.	CD8+ TCR-Vα 7.2^+ $CD161^-$ (sorted 488 cells; used 1/15 of cDNA)
6.	$CD3^+$ TCR-Vα 7.2^- Negative Control cDNA
7.	Reverse Transcriptase Enzyme Negative Control
8.	DNA Polymerase Enzyme Negative Control

Figure 38 *Legend*

FACS was performed on PBMC from a healthy donor. RNA was extracted from the sorted T-cells which was then reverse transcribed to make cDNA and PCR performed. T-cell cDNA of $CD3^+$ TCR-Vα$7.2^{+/-}$ was also isolated to serve as positive and negative controls. The result of agarose gel electrophoresis on the PCR products is shown.

Chapter 5: Discussion

In the past, few reports have produced detailed analyses of the lymphocytic infiltrate present in pancreatic tissue allied with concurrent examination of peripheral blood lymphocytes in chronic pancreatitis patients. Published evidence increasingly suggests a role for lymphocytes in the pathogenesis of CP which led to the generation of four hypotheses: 1) The peripheral blood of patients with chronic pancreatitis is comprised of different proportions of T-cell subsets; 2) Specific T-lymphocyte subsets are enriched in chronic pancreatitis tissue; 3) The proportion of T-regulatory cells to T-helper cells is skewed in the pancreatic tissue and peripheral blood of patients with chronic pancreatitis; and 4) Mucosal-associated invariant T-cells infiltrate chronically inflamed pancreatic tissue and are accordingly depleted in peripheral blood.

I therefore firstly aimed to develop a reproducible technique to isolate live lymphocytes from pancreatic tissue to allow their detailed study. The successful completion of this primary task facilitated the research project. Although there are few published studies to compare my technique to, the yields of lymphocytes obtained from pancreatic tissue were sufficient and allowed their further characterisation. The progressive development of this new technique was fundamental to the research and will allow future immunological studies of pancreatic inflammatory processes to be undertaken.

Having developed a reproducible technique to extract lymphocytes from pancreatic tissue I performed a phenotypic analysis of intrapancreatic and peripheral blood lymphocytes in patients with CP to identify T-lymphocytes and mucosal-associated invariant T-cells, and to functionally characterise CD4+ T-helper cell lineages (Th1/2/17) and T-regulatory cells. As the logistics of completing such a large number of analyses at one time on samples collected were insurmountable, I decided that all samples should be cryopreserved for storage and then examined in the future. To determine whether this would have an effect on specific T-lymphocyte populations I conducted a short experimental protocol to compare the analysis of fresh and frozen samples. This not only provided an opportunity to refine the required techniques but also to determine future methodologies. Although the number of samples examined in this manner was small it gave an indication of potential problems and re-enforced the notion that a consistent methodology of comparative analysis was required.

The subsequent work conducted generated a significant number of results and some novel and interesting findings. The study of both T-helper and T-regulatory lymphocytes in CP was the first of its kind, as was the examination of MAIT cells. In addition the study produced several findings that have contradicted some of the published literature as well as providing numerous potential future avenues of research.

PERIPHERAL BLOOD T-CELL SUBSET ANALYSIS IN CHRONIC PANCREATITIS AND HEALTHY CONTROLS

The examination of peripheral blood revealed a trend towards a lower proportion of CD4+ and higher proportion of CD8+ T-cells in patients with CP compared to controls. This led to a trend for an altered CD4:CD8 ratio, although no difference existed in the proportion of DN T-cells found, or in the proportion of CD8+CD161+ cytotoxic T-cells identified. Previous authors have produced discordant results of T-cell subsets: Whilst early publications demonstrated a reduction in the percentage of CD8+ T-cells in CP patients compared to controls,[134] others subsequently found a reduction of CD4+ T-cells,[136] or an increase in the total number of CD4+ T-cells with no change in CD8+ T-cells.[138] These studies consisted of similarly sized patient groups. Therefore to definitively elucidate whether any true differences exist between the proportions of T-cell subsets present in the peripheral blood of patients with CP, recruitment and analysis of a significantly larger patient group is required.

In subtle contradiction to the findings by Grundsten *et al*, who demonstrated an increase in peripheral blood $CCR7^{+}CD45RA^{-}$ central memory T-cells in CP patients compared to healthy controls,[137] I found no difference in the proportion of $CD45RO^{+}$ memory to $CD45RO^{-}$ naïve T-cells in CP. The reasons for this may be twofold: In their study, half of the patients had severe disease that necessitated surgery; and they used an additional cell marker (CCR7) to identify central memory T-cells. My experimental design lacked the specificity to detect changes in particular memory T-cell populations, such as those expressing CCR7 which functions to guide cells out of tissues, into lymphoid organs and then back into the circulation.[213, 214] I therefore conclude that my findings are representative of the fact that patients with CP do not have an increased proportion of total $CD45RO^{+}$ memory T-cells in their peripheral blood, however specific populations, such as effector memory or central memory T-cells may be altered.

An interesting finding that merits further consideration and exploration was that the peripheral blood of patients with chronic pancreatitis has a trend for a lower proportion

of $CD8^+$ integrin $\alpha4\beta7^+$ T-cells compared to controls. This trend was not evident for $CD4^+\alpha4\beta7^+$ T-cells. A possible explanation for this is that there is a specific enrichment of $CD8^+\alpha4\beta7^+$ T-cells in inflamed pancreatic tissue which leads to their corresponding decrease in peripheral blood. An alternative explanation could be that as small bowel bacterial overgrowth is common in CP the $CD8^+\alpha4\beta7^+$ T-cells accumulate there instead.[178] As the chemokine and integrin signature of exocrine pancreas infiltrating lymphocytes has yet to be identified, it would be of great interest to define whether integrin $\alpha4\beta7^+$ T-cells were found in the pancreatic tissue of CP patients in significant numbers and whether this is the defining pancreatic tissue homing signature. In support of this hypothesis, mouse models of diabetes demonstrate infiltration by T-lymphocytes expressing integrin $\alpha4\beta7$.[121-124] This therefore represents an important unexplored aspect of pancreatic disease that could yield potential for therapeutic intervention. Monoclonal antibodies directed against integrin $\alpha4\beta7$ are commercially available as the drug Natalizumab and have been successfully used for the treatment of inflammatory bowel disease.[219] If the integrin signature of exocrine pancreas infiltrating lymphocytes was shown to be integrin $\alpha4\beta7$ it would justify analysis of whether Natalizumab could be used to treat inflammatory pancreatic disease.

TISSUE INFILTRATING T-CELLS IN CHRONIC PANCREATITIS

Examination of chronic pancreatitis tissue using immunohistochemistry and flow cytometry revealed that it contains significantly increased numbers of CD3+ T-cells compared to normal pancreatic tissue. These T-cells are likely comprised of equal proportions of CD4+ and CD8+ T-cells. There was however a discrepancy in results depending on the methodology employed: When pancreatic tissue was assessed using flow cytometry T-cell subsets were divided into roughly equal proportions of CD4+ and CD8+ cells (CD4:CD8 = 1.1), however this ratio was 0.42, i.e. CD4<CD8, when assessed using immunohistochemistry. Similar disparities have been found between previously published works: Flow cytometry analysis suggests an equal ratio of CD4:CD8 in pancreatic tissue whereas immunohistochemical methods have suggested either CD4 predominance or an equal ratio.[39, 130-133] My results therefore reflect the inherent difficulties faced with examining pancreatic tissue.

During the development of the protocol to isolate lymphocytes from pancreatic tissue I noted that CD4 could be cleaved from the surface of T-cells during the digestion of pancreatic tissue. Furthermore it was often difficult to obtain good immunohistochemical staining of CD4+ T-cells in GMA embedded chronic pancreatitis tissue. Both of these technical issues could have skewed my results. However the

reproducible good quality of the flow cytometry data, the larger volume of tissue examined using flow cytometry and its higher sensitivity in detecting total CD4 counts, lead me to conclude that its results are most likely to be representative and accurate, and that the T-cell infiltrate found in chronic pancreatitis is composed of equal proportions of CD4+ and CD8+ T-cells.

In the patients whose pancreatic tissue was examined, comparison of the proportions of T-cells in peripheral blood was made. This revealed that the proportion of CD4+ T-cells found in the tissue of CP patients is significantly lower than that found in peripheral blood, however no difference exists when CD8+ and double negative T-cells are examined in the same manner. This discrepancy is likely to be the result of statistical error induced by the small sample size resulting in failure to detect a true difference between the two compartments. It is probable that expansion of this study would produce the expected result i.e. the finding of an increased proportion of CD8+ and DN T-cells in pancreatic tissue compared to blood.

T-HELPER AND T-REGULATORY CELLS IN CHRONIC PANCREATITIS

Analysis of peripheral blood revealed that a significantly higher proportion of Th1, Th2 and Th17 cells was found in chronic pancreatitis patients compared to controls. Previous authors have suggested polarisation of the peripheral blood T-helper cell phenotype towards a Th1 dominant response.[136] However this study measured total IFN-γ production by CD3+ T-cells. As CD8+ T-cells can produce significant quantities of IFN-γ my methodology was more precise as it specifically examined T-helper cells for IFN-γ production, as well as IL-13 and IL-17. Further analysis of peripheral blood also showed that a significantly higher proportion of Treg cells were found in chronic pancreatitis patients compared to controls. Other studies have concurred that Treg cell numbers are increased in the peripheral blood of CP patients, whereas another found no difference.[139, 140]

This study is the first to definitively demonstrate that Th1 cells are increased in number and that other T-helper cell lineages and T-regulatory cells are augmented. This heightened T-helper cell population is not explained by an increase in the number of activated $CD4^{+}CD25^{+}$ cells present. This suggests that activated $CD4^{+}CD25^{+}$ T-cells do not drive inflammation in chronic pancreatitis, or lead to the increased numbers of Treg cells seen (that are $CD4^{+}CD25^{+}FoxP3^{+}CD127^{-/lo-}$). It does however infer that the increased numbers of T-helper cells present in the peripheral blood of CP patients may orchestrate and perpetuate the inflammatory response.

These changes may represent a fundamental tenet of chronic pancreatitis; namely that cytokine secreting T-cells are globally more prevalent in this disease. The fact that Treg cells are also increased in number suggests that the equilibrium between inflammation and repair has not been established. Inflammatory processes require control mechanisms to prevent tissue destruction becoming pervasive. This is one of the central roles of Treg cells whereby their up-regulation dampens inflammatory responses. As neither pro-inflammatory Th1 and Th17 cells, or anti-inflammatory Treg cells, are predominant in CP; and the majority of patients in the study had alcoholic CP but had stopped drinking, it suggests that an occult on-going stimulus potentiates the disease preventing tissue repair resulting in organ fibrosis. It appears therefore that the blood of CP patients is primed to respond non-specifically to inflammatory stimuli.

It was intriguing to find that the peripheral blood of patients who consume excess alcohol contains significantly more Th1 cells than non-drinkers. This was true for all patients when examined (both CP and "healthy controls") and for those with CP. This might be related to the fact that alcohol increases gut permeability causing high circulating levels of lipopolysaccharide which in turn drives the generation of Th1 cell responses.[169-171] These combined observations of the T-helper phenotype may help to explain the perpetuation of pancreatic inflammation and the pathogenesis of CP.

The analysis of CP tissue for T-helper and T-regulatory cells represented another novel aspect of the study. I found that the CD4+ T-helper cell infiltrate in chronic pancreatitis was predominantly comprised of IFN-γ^+ Th1 cells when analysed using flow cytometry. IL-17^+ Th17 cells were also seen, including a small but discernible number of dual secreting IFN-γ^+IL-17^+ Th17 cells. No IL-13+ Th2 cells were identified and few or no FoxP3^+ Treg cells. When pancreatic tissue was analysed using immunohistochemistry IFN-γ^+ cells were only identified in CP tissue and not controls, and $Th2^+$, $Th17^+$ and FoxP3^+ cells were not identified in any pancreatic tissue. T-helper cells infiltrating CP tissue therefore seem polarised to a Th1 phenotype with additional Th17 cell presence. The absence of IL-17 immunohistochemical staining is most likely explained by the fact that cytokines are not stored in large quantities and Th17 cells are scarce, thus they are difficult to detect unless large quantities of tissue are examined. This problem could now be overcome as new antibodies for use in immunohistochemistry have become available that allow identification of the transcription factors that govern specific T-helper cell differentiation. These however have yet to be tested in GMA embedded tissue. Nevertheless the results from the flow cytometry analysis provide enough

evidence for the polarisation towards Th1, and they are supported by the findings of IFN-γ^+ cells in GMA embedded tissue.

In other inflammatory diseases there is frequently a polarisation of T-helper lymphocyte responses towards either a Th1 or Th2 phenotype. However inflammatory diseases may also involve bimodal immune reactions which initially begin with Th1 and Th17 dominant responses, and then become Th2 and Treg mediated if the immune stimulus persists. This switch to an anti-inflammatory and suppressive response exists to prevent excessive damage to host tissues and promote wound healing.[103] Chronic pancreatitis is a disease characterised by tissue fibrosis, which is generally associated with a Th2 dominant response.[101, 102] The clear predominance of a Th1 response in the CP tissue I examined may be explained by a number of different possibilities: the tissue examined is not characteristic of CP; the patchy nature of CP skewed the results; or that the simple Th1/Th2 paradigm is not applicable to CP. This merits discussion in more detail:

Firstly it is conceivable that I examined tissue that was in an inflammatory rather than a "healing" fibrotic phase. This could have been a manifestation of the patchy nature of CP. Alternatively it may be that destruction of pancreatic tissue involves significant numbers of Th1 cells but tissue fibrosis only requires a small number of Th2 cells. In addition a number of other possibilities exist. Whereas the most well described Th2 dominant diseases are highly organ specific (e.g. asthma and schistosomiasis)[103, 220] it is entirely feasible that this simple dichotomy does not exist in CP. This potential explanation is corroborated by inflammatory bowel diseases: Whilst Crohn's disease is associated with tissue fibrosis it is also characterised by Th1 predominance with an important additional role played by Th17 cells;[51, 70] Ulcerative colitis on the other hand does not fit neatly into the Th1/Th2 paradigm at all.[221]

Another explanation for the Th1/Th2 paradigm could be that patients with CP exhibit a genetic predisposition to Th1 mediated disease. It is known that overexpression of GATA-3 predisposes to Th2 mediated diseases such as allergic asthma, whereas activation of T-bet appears to be an essential step for Th1 mediated mucosal diseases such as Crohn's disease.[220] Examination of polymorphisms in these transcription factors in the context of CP has potential to produce interesting results therefore.

The interaction of pancreatic stellate cells and infiltrating lymphocytes could also be significant in the pathogenesis of CP. PSC are stimulated by IL-13, an archetypal Th2

cell cytokine, IL-10, which is often produced by Treg cells, and TNF-α, a potent pro-inflammatory cytokine.[152, 155] It is possible therefore that it is the persistent activation of PSC by cytokines released from different T-cells during distinct phases of acute or chronic inflammation that drives the progression from acute to chronic pancreatitis. Whether Th1, Th2, Th17 or Treg cells drive this becomes irrelevant as the continual activation of PSC allows the disease to progress regardless.

It is also imaginable that as the significant majority of CP patients are alcoholics this may alter their T-helper cell homeostasis. Certainly the high numbers of Th1 cells I found in the blood of CP patients who continue to drink alcohol would support this postulate. In particular the majority of CP tissue examined (>75%) came from patients with an alcoholic aetiology. Clearly further evaluation of the interplay between alcohol and T-cell function in CP is required. It should be noted however that similar histopathological changes are seen in pancreatic tissue regardless of the aetiology and the disease reaches a common immunological stage beyond which it appears to progress as a single distinctive entity.[39] This supports the theory of a common immune mediated tissue destructive process. As obtaining human chronic pancreatitis tissue early in the disease course is not possible new animal models are the only way this can be examined further.

Finally one should not overlook the possibility that T-helper cells are not actually involved in the pathogenesis of CP. The tissue examined in all studies represents the end-stage of the disease and thus at best I can merely make an observation that Th1 and Th17 cells are present. It is possible that the observed inflammatory cell infiltrate is associated with tissue ischaemia caused by de-vascularisation related to fibrosis. Subsequent bacterial infiltration of dying hypoxic tissue and release of free radicals allied with tissue necrosis may have then promoted the lymphocyte infiltrate. In essence therefore it is important to remember that this work was an observational study and did not provide evidence that the lymphocytes have a functional role in the pathogenesis of CP.

MAIT CELLS IN CHRONIC PANCREATITIS

Mucosal-associated invariant T-cells have not been the focus of investigation in chronic pancreatitis before. As MAIT are found in significant numbers in the gut,[222] small bowel bacterial overgrowth is common in CP,[178] and bacterial translocation from the gut is promoted by alcohol,[169] I hypothesised that MAIT were specifically enriched in CP tissue. I therefore examined pancreatic tissue and blood and found that there was a

significant enrichment of double negative MAIT cells in the pancreatic tissue of CP patients compared to peripheral blood. However no such difference existed for CD8+ MAIT cells nor was there any difference observed in the number of MAIT cells in the peripheral blood of CP patients and controls. The understanding of MAIT cells is very much in its infancy and thus far we only know that they have a role in the identification and elimination of microbial pathogens.[111, 112] The detection of MAIT cells in CP tissue therefore suggest that they are recruited not only to a site of inflammation but possibly also pancreatic infection. How the bacteria may have entered pancreatic tissue can be debated but it is most likely related to bacterial translocation from the gut. The common occurrence of small bowel bacterial overgrowth in CP patients supports this notion.[178]

It is also feasible however that MAIT have infiltrated CP tissue due to aberrant homing mechanisms as they are usually found at mucosal surfaces, particularly the gut.[106] The potential regulatory capability of MAIT cells could also explain their infiltration into inflamed pancreatic tissue as they have been shown to have inhibitory activity against IFN-γ secreting T-cells in MS patients.[113] As I have shown that Th1 cells infiltrate CP tissue in significant numbers, MAIT could therefore be present in a suppressive and regulatory capacity. As MAIT have been shown to secrete a mixture of pro- and anti-inflammatory cytokines following TCR ligation, including IFN-γ, IL-4, IL-5, IL-10, IL-17 and Granzyme-B, it is difficult to pin-point their exact role.[109, 110] Research into MAIT cells as a whole represents a fascinating field of immunology and their role in CP should be evaluated further.

CONCLUSION

Chronic pancreatitis remains a disease that is difficult to diagnose, treat and understand. As it only becomes clinically apparent following significant tissue damage it presents an ongoing challenge in terms of earlier diagnosis and scientific investigation. Surgery for CP is undertaken infrequently even in large pancreatic surgical centres thus tissue is scarce and that obtained only represents the end-stage of the disease. Animal models can help fill the gap but financial, ethical and mechanistic concerns still hinder progress. Our understanding of the pathogenesis of the disease has progressed over the last two decades but still significantly lags behind its contemporaries, such as liver cirrhosis, in terms of publications and centres undertaking research. This study has produced some interesting findings that have characterised the lymphocyte immune reaction in CP in more detail. In particular the T-helper and MAIT cell results represent exciting discoveries and a number of new questions have been generated. Whilst this phenotypic analysis lacks any true functional data it provides a basic

framework for future study into this fascinating topic. Improvement of our understanding of the pathogenesis of CP will hopefully allow the development of new treatments in the future. These may then improve the outcome of this painful, debilitating disease.

POTENTIAL FUTURE WORK

If this study had been extended the next work I would have undertaken would have involved the stimulation of isolated CP infiltrating T-cells with homogenates of chronic pancreatitis and normal pancreatic tissue, to assess the cytokine secretion profile of the T-cells present. Furthermore attempts would have been made to examine the cytokines produced by MAIT cells from CP patients and their interactions with pancreatic stellate cells and Th1 cells.

Analysis of more patients would be needed to determine if a statistical difference in the proportion of T-cell subsets and $CD8^+$ integrin $\alpha4\beta7^+$ T-cells exists in the peripheral blood of CP patients, or whether the CD4:CD8 ratio is altered in tissue compared to blood. These all represent useful future lines of study.

Identification of the homing markers that promote lymphocyte infiltration into pancreatic tissue represents a further exciting potential area of research. Integrin α4β7 seems to be a likely starting point for this and may serve as a good target for therapeutic blockade. Analysis of isolated CP infiltrating T-cells using gene arrays would allow preliminary results to be gathered allowing validation experiments to then be performed.

Appendix

POSTER PRESENTATIONS

- **International Association of Pancreatology Annual Meeting 2011.**
  Development of a Technique to Isolate T-Lymphocytes from Pancreatic Tissue.
  Jupp J, Fine D, Johnson C, Gadola S.

- **British Society of Gastroenterology Annual Meeting 2011.**
  Identification of Mucosal-Associated Invariant T-Cells in Tissue and Blood of Patients with Chronic Pancreatitis.
  Jupp J, Fine D, Johnson C, Lantz O, Gadola S.

- **Digestive Disease Week 2011.**
  Mucosal-Associated Invariant T-Cells Infiltrate Pancreatic Tissue in Patients with Chronic Pancreatitis.
  Jupp J, Fine D, Johnson C, Lantz O, Gadola S.

- **European Pancreatic Club Meeting 2011.**
  Mucosal-Associated Invariant T-Cells Infiltrate Pancreatic Tissue in Chronic Pancreatitis.
  Jupp J, Fine D, Johnson C, Lantz O, Gadola S.

ORAL PRESENTATIONS

- **Digestive Disorders Federation Meeting 2012.**
  Characterisation of T-Helper and T-Regulatory Cells in Chronic Pancreatitis.
  Jupp J, Fine D, Johnson C, Gadola S.

PATIENT CONSENT FORM

Dr David Fine,
Liver & Pancreatic Research Group,
Infection, Inflammation and Immunity Division,
Southampton University School of Medicine.

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Southampton General Hospital, Mailpoint 255,
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Tel +44 (0)23 8079 4153
Fax +44 (0)23 8079 5203
E-mail: David.Fine@suht.swest.nhs.uk

CONSENT FORM

STUDY TITLE: IMMUNOLOGY OF PANCREATIC DISEASE

REC submission No: 09/H0604/105

Patient Identification Number for this Trial ☐

Please Initial Box

1. I confirm that I have read and understand the information sheet dated ..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the research group, by regulatory authorities, or by Southampton University Hospitals NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to the storage and use of my donated tissue samples and/or blood in this study. ☐

5. I agree to the storage of my donated tissue samples and/or blood for future research. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Version 2 - 03/11/2009
When completed: 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

PATIENT INFORMATION SHEET

Dr David Fine,
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Infection, Inflammation and Immunity Division,
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PARTICIPANT INFORMATION SHEET

STUDY TITLE: IMMUNOLOGY OF PANCREATIC DISEASE

Protocol Reference No: Version 2
REC Submission No: 09/H0604/105

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of the study and what will happen to you if you take part.
Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

The departments of medicine and surgery at the University of Southampton are investigating how the immune system responds to injury of the pancreas and/or the development of cancer. In order to do this we are studying samples of human pancreatic tissue and pancreatic lymph nodes (parts of the immune system that are important for its functioning) that have been removed during operations on the pancreas. We are also collecting samples of blood that will allow us to compare the immune cells in the blood to those that have entered the pancreas and the lymph nodes. We shall be using a number of laboratory techniques to analyse the specimens to examine specific characteristics of the immune cells. This will allow us to understand how pancreatic disease develops and therefore how we may better treat the conditions.

Why have I been invited?

You have been invited for one of two reasons. 1) You may be about to undergo an operation that will involve the removal of part of the pancreas and adjacent lymph nodes. If you are willing to consent to our study, we will take a small portion of the tissue that is removed during your operation. This will not require any extra tissue to be removed as we would use part of the tissue that is going to be taken from your pancreas anyway. There will be some instances when pancreatic tissue is removed due to a problem with organs adjacent to the pancreas. This pancreatic tissue is normal

but is also very valuable to study. You may therefore be asked to enter the study even if you have no problems with your pancreas. 2) You may however have been selected to donate a sample of blood as you have pancreatic disease that does not require surgery or you are a healthy adult that will serve as a control to allow comparison between disease and healthy states.

Do I have to take part?

No, it is up to you to decide whether you wish to take part or not. We will describe the study and go through this information sheet, which we will then give to you to keep. If you agree to take part, we will ask you to sign a consent form. If you do agree to take part you are free to withdraw at any time, without giving a reason. The standard of the care which you receive will be the same whether or not you take part in this study.

What will happen to me if I take part?

If you are not having surgery then we will take blood from you only.

If you are due to have surgery and you agree to take part, a small piece of pancreatic tissue and a small piece of pancreatic lymph node will be taken from the surgical specimen removed. In addition a sample of blood will be taken. If you do not wish to provide a blood sample now then it can be taken during the operation.

Once the surgical specimen and/or blood have been obtained, you will not be required to undergo any further investigation or follow up for the purposes of this study. The scientific procedures used in the study are not diagnostic and will not be used to determine further treatment. They are purely to aid our understanding of the underlying disease process. You will therefore not be exposed to any extra risk in addition to the risks of your treatment. No other inconvenience is anticipated.

What are the possible benefits of taking part?

The study will have no direct benefit to you, but the information we get from this study will help our understanding of pancreatic disease. The tissue and blood samples will be used to examine how the immune system responds to pancreatic injury and the development of cancer. This may help to develop new methods of treatment in the future.

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice so that all the information about you will be handled in the strictest confidence.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. If you withdraw from the study, we will destroy all identifiable samples, but we will use the data up to your withdrawal. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

If you participate in the study, you will be free to withdraw at any time without having to give any reason. Your decision to withdraw will not affect your medical care in any way.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (Dr James Jupp: 023 8079 6663; Dr David Fine 023 8079 4153). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (or private institution). Details can be obtained from the hospital.

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Procedures for handling, processing and storing confidential data will follow the Data Protection Act 1998. We will collect data relevant only to our study; this will include your age, sex, medical history and any medication you are currently on or have previously taken. This data will be coded so that you cannot be identified. It will then be stored securely and made available only to members of the research team. Any identifiable data collected will be destroyed at the end of research study.

What will happen to any samples I give?

If you are due to have surgery then pancreatic and lymph node tissue will be removed as part of the operation. We will take a small piece of each of these tissues. This will not have any affect on the amount of tissue required to make a diagnosis and will not involve taking more tissue than is required for the operation you need. The blood sample can be collected at a point convenient to you up until the end of your operation.

If you are not due to have surgery then only a blood sample will be taken, at a time convenient to you.

All samples will be coded both for storage and their use in any experiments so that you cannot be easily identified.

Blood and tissue samples will be processed in a secure laboratory. The samples will be available for researchers in the Liver and Pancreatic Research Group of The University of Southampton School of Medicine to study. We will store the tissue samples in paraffinised blocks, glycol methacrylate resin or by freezing to -80°C to preserve the integrity of the specimens. These different methods allow a number of experimental techniques to be used to examine the tissues in detail. In particular we shall assess: the immune cells in detail; communication between immune cells and specific resident pancreatic cell populations; and the amount of specific proteins and genetic material present. We will use sophisticated laboratory methods which have been developed over many years and validated by many research groups around the world to examine the tissue and blood samples. The information obtained from the samples is unlikely to yield any information that will be individually significant.

We will request your consent to collect any blood and tissue samples for use in this research project. We will also ask your consent for these blood and tissue samples to be stored for future research. Any samples stored may be made available to members of the Liver and Pancreatic Research Group or other research groups within the University of Southampton if they are granted approval from a Regional Ethics Committee. If future researchers are granted ethical approval then any tissue used will be made anonymous such that you cannot be identified under any circumstance.

What will happen to results of the research study?

The results of any research are likely to be published in the scientific literature. There will no means of identifying individuals within this.

Who is organising and funding the research?

The research has been organised by the Liver and Pancreatic Group of the Division of Infection, Inflammation and Immunity at The University of Southampton School of Medicine and is being led by Dr. David Fine, consultant gastroenterologist and honorary senior lecturer, in collaboration with the department of surgery. This research is being supported by Spire Southampton Healthcare, a private hospital that has entered into an agreement with The University of Southampton. Under this arrangement investigators work for Spire Southampton Healthcare as Resident Medical Officers for 1-2 days per week and spend the rest of their time working in the laboratory. Spire Southampton Healthcare donates money to The University of Southampton to support laboratory work but has no control over the research conducted and no rights to the results, which belong to the researchers and The University of Southampton.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Oxfordshire Research Ethics Committee A.

Further Information:

If you have any questions please fell free to ask the study doctors directly at Southampton General Hospital: Dr. James Jupp - telephone number 023 8079 6663 or Dr. David Fine – telephone no: 023 8079 4153.

Alternatively for independent advice you can contact 'Involve', a national advisory group, funded by the National Institute for Health Research (NIHR), whose role is to support and promote active public involvement in NHS, public health and social care research. Website: www.invo.org.uk/index.asp. Telephone: 02380 651088.

Many thanks for your co-operation.

SOLUTION FORMULAE FOR ISOLATION OF MONONUCLEAR CELLS FROM PANCREATIC TISSUE AND PERIPHERAL BLOOD

Complete Medium

Dulbeccos Modified Eagles Medium (DMEM) (4.5g/L glucose, no L-glutamine) (Lonza)
Foetal Calf Serum (FCS) (heat inactivated) (10%) (Invitrogen)
Penicillin / Streptomycin (1%) (Lonza)
L-Glutamine (1%) (Lonza)

Lymphocyte Isolation Buffer

Phosphate Buffered Saline without calcium & magnesium (PBS) (Lonza)
Foetal Calf Serum (heat inactivated) (10%) (Invitrogen)
EDTA (1mM) (0.292 mg/L) (Sigma Aldrich)
N-Acetyl Cysteine (100μM) (16.3 mg/L) (Sigma Aldrich)
Ascorbic acid (100μM) (17.6 mg/L) (Sigma Aldrich)
Pefabloc (400μM) (100 mg/L) (Roche)
D-Glucose (4.5 g/L) (Sigma Aldrich)

RPMI Lymphocyte Culture Medium

RPMI 1640 without L-Glutamine (Lonza)
L-glutamine (2 mM) (1%) (Lonza)
Sodium pyruvate (100 mM) (1%) (Lonza)
Penicillin/Streptomycin (1%) (Lonza)
Foetal Calf Serum (heat inactivated) (10%) (Invitrogen)

RPMI Wash Solution

RPMI 1640 (Gibco)
Foetal Calf Serum (heat inactivated) (10%) (Invitrogen)

SOLUTION FORMULAE FOR FLOW CYTOMETRY

FACS Buffer

Bovine Serum Albumin (1%) (Sigma Aldrich)
Phosphate Buffered Saline (Lonza)
Sodium Azide (0.1%) (Sigma Aldrich)

SOLUTION FORMULAE FOR IMMUNOHISTOCHEMISTRY

Blocking Medium

Dulbecco's Modified Eagles Medium 80ml (Sigma Aldrich)
Foetal Calf Serum 20ml (PAA)
Bovine Serum Albumin 1g (Sigma Aldrich)

GMA Embedding Solution

GMA solution A 5ml (Polysciences)
GMA solution B 125µl (Polysciences)
Benzoyl peroxide 22.5mg (Polysciences)

Mayer's Haematoxylin

Haematoxylin 1g (Fisher Scientific)
Distilled water 1000ml
Potassium alum 50g (Fisher Scientific)
Citric acid 1g (Fisher Scientific)
Chloral hydrate 50g (Fisher Scientific)
Sodium iodate 0.2g (Fisher Scientific)
The haematoxylin, potassium alum and sodium iodate are dissolved in the distilled water at room temperature overnight. The chloral hydrate and citric acid are added and the mixture is then boiled in a fume extraction hood for 5 minutes, then cooled and filtered.

ANTIBODIES USED IN FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY

Table 28: Manufacturer Information on Antibodies and Dyes used for Flow Cytometry

Antibody / Dye + Fluorochrome	Antibody Class	Manufacturer	Clone	Concentration (µg / µl)	Volume / Test (µl)
Anti-CD161-FITC	Mouse IgG1	BD Bioscience	DX12	0.05	10
Anti- Integrin α4-PE	Mouse IgG1	Abcam	44H6	0.1	5
Anti-CD4-PerCP-Cy5.5	Mouse IgG1	BD Bioscience	RPA-T4	0.1	2
Anti-CD8-APC-Cy7	Mouse IgG1	BD Bioscience	SK1	0.006	2
Anti- Integrin β7-APC	Rat IgG2a	BD Bioscience	FIB504	0.0125	10
Anti-CD3-PE-Texas Red	Mouse IgG2a	Invitrogen	S4.1	0.1	2
Anti-CD45RO-PE-Cy7	Mouse IgG2a	BD Bioscience	UCHL1	0.05	2
Anti-IL-13-FITC	Mouse IgG1	eBioscience	PVM13-1	0.05	2
Anti-IL-17-PE	Mouse IgG1	eBioscience	eBio64DEC17	0.05	2
Anti-IFNγ-PE-Cy7	Mouse IgG1	eBioscience	4S.B3	0.025	2
Anti-CD127-FITC	Mouse IgG1	BD Bioscience	HIL-7R-M21	0.05	10
Anti-CD25-PE	Mouse IgG1	eBioscience	BC96	0.025	2
Anti-FoxP3-APC	Rat IgG2a	eBioscience	PCH101	0.1	2
Anti-TCR Vα7.2-Biotin	Mouse IgG1	N/A †	N/A	1.0	1
Streptavidin-PE	N/A	BD Bioscience	554061	--	1
Live/Dead Fixable Violet Dye	N/A	Invitrogen	L34955	--	0.5

† Anti-TCR Vα7.2-Biotin antibody kindly donated by Olivier Lantz, Institut Curie, Paris

Table 29: Manufacturer Information on Isotype Control Fluorochrome Conjugated Antibodies

Antibody Target	Isotype Control Antibody	Manufacturer	Clone	Conc. (µg / µl)	Volume / Test (µl)
Integrin α4 CD25	Mouse IgG1 PE	eBioscience	12-4714	0.1	5
CD4	Mouse IgG1 PerCP-Cy5.5	BD Bioscience	550795	0.2	1
CD8	Mouse IgG1 APC-Cy7	BD Bioscience	557873	0.05	0.3
CD3	Mouse IgG2a PE-Texas Red	Invitrogen	MG2a17	0.1	2
CD45RO	Mouse IgG2a PE-Cy7	BD Bioscience	560608	0.05	2
CD127 CD161 IL-13	Mouse IgG1 FITC	eBioscience	17-4321	0.05	10
IL-17	Mouse IgG1 PE	eBioscience	12-4714	0.1	1
IFNγ	Mouse IgG1 PE-Cy7	eBioscience	25-4714	0.05	1
FoxP3 Integrin β7	Rat IgG2a APC	eBioscience	17-4321-41	0.025	5

Table 30: Antibodies Tested for Paraffin Immunohistochemistry

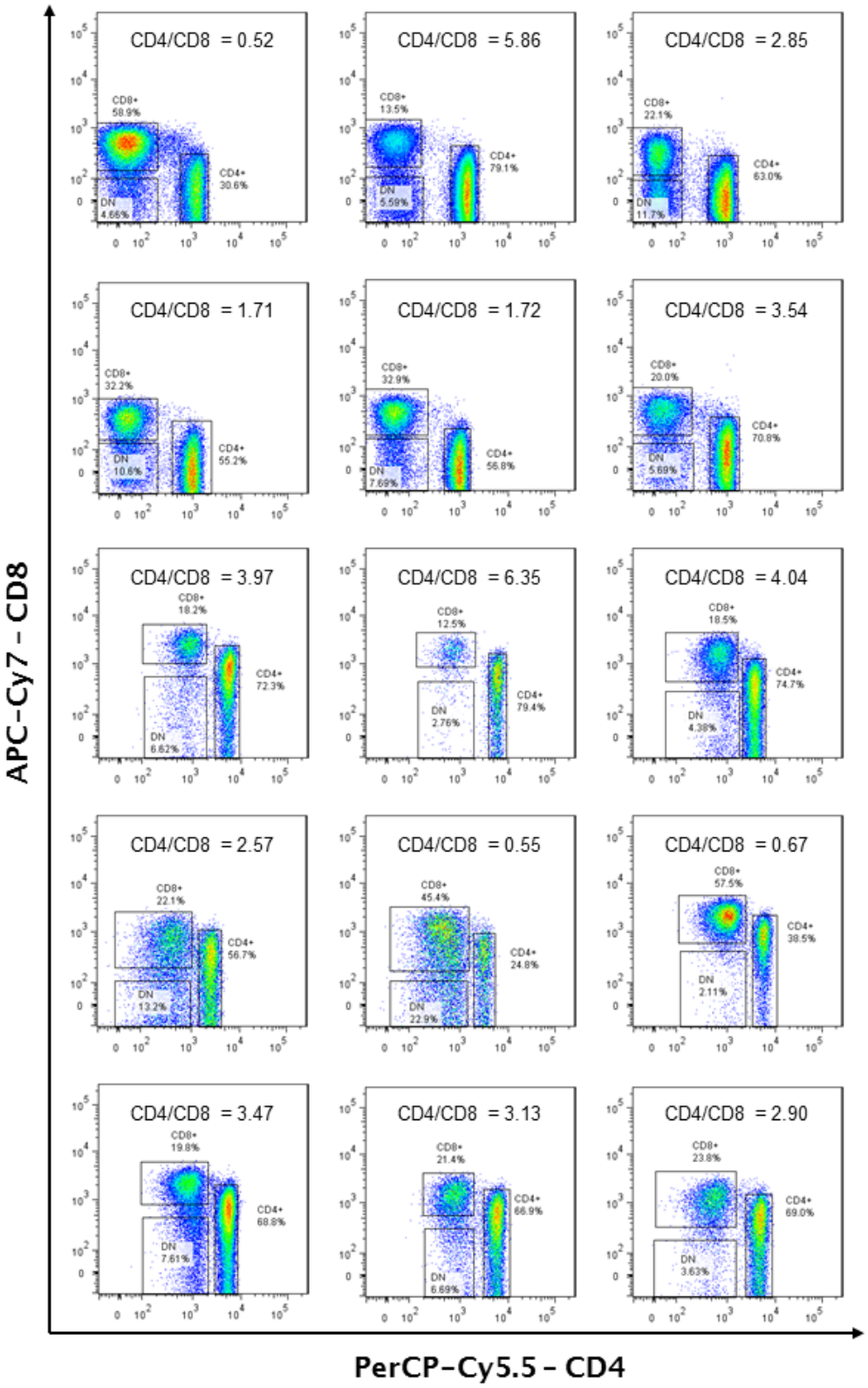
Antigen	Antibody Manufacturer	Clone	Antibody Type	Positive Control
Microwave Heat Mediated EDTA Buffer Antigen Retrieval				
CD3	Dako	F7.2.38*	Monoclonal Mouse IgG1	Tonsil
CD8	Dako	C8/144B*	Monoclonal Mouse IgG1	Tonsil
Microwave Heat Mediated Citrate Buffer Antigen Retrieval				
FoxP3	AbCam	236A/E7	Monoclonal Mouse IgG1	Tonsil
Unable to Produce Reliable and Reproducible Staining				
CD4	Novocastra	NCL-L-CD4-1F6*	Monoclonal Mouse IgG1	Tonsil
CD4	AbCam	BC/1F6*	Monoclonal Mouse IgG1	Tonsil
CD4	AbCam	Ab67480	Monoclonal Mouse IgG1	Tonsil
CD103	AbCam	2G5.1	Monoclonal Mouse IgG2a	Jejunum
CD161	AbCam	B199.2	Monoclonal Mouse IgG2b	Tonsil
CD161	BD Bioscience	DX12	Monoclonal Mouse IgG1	Tonsil
IL-17	R&D	AF-317-NA	Polyclonal Goat IgG	Tonsil

* These antibodies had been tested by the manufacturer and were suitable for paraffin immunohistochemistry. It should be noted that the anti-CD4 1F6 clones gave patchy, inconsistent staining. Correspondence with the manufacturers revealed no problems with the batches. Discussion with other groups in the research division uncovered similar problems such that many had abandoned staining for CD4 in paraffin embedded tissues altogether and processed all specimens into GMA.

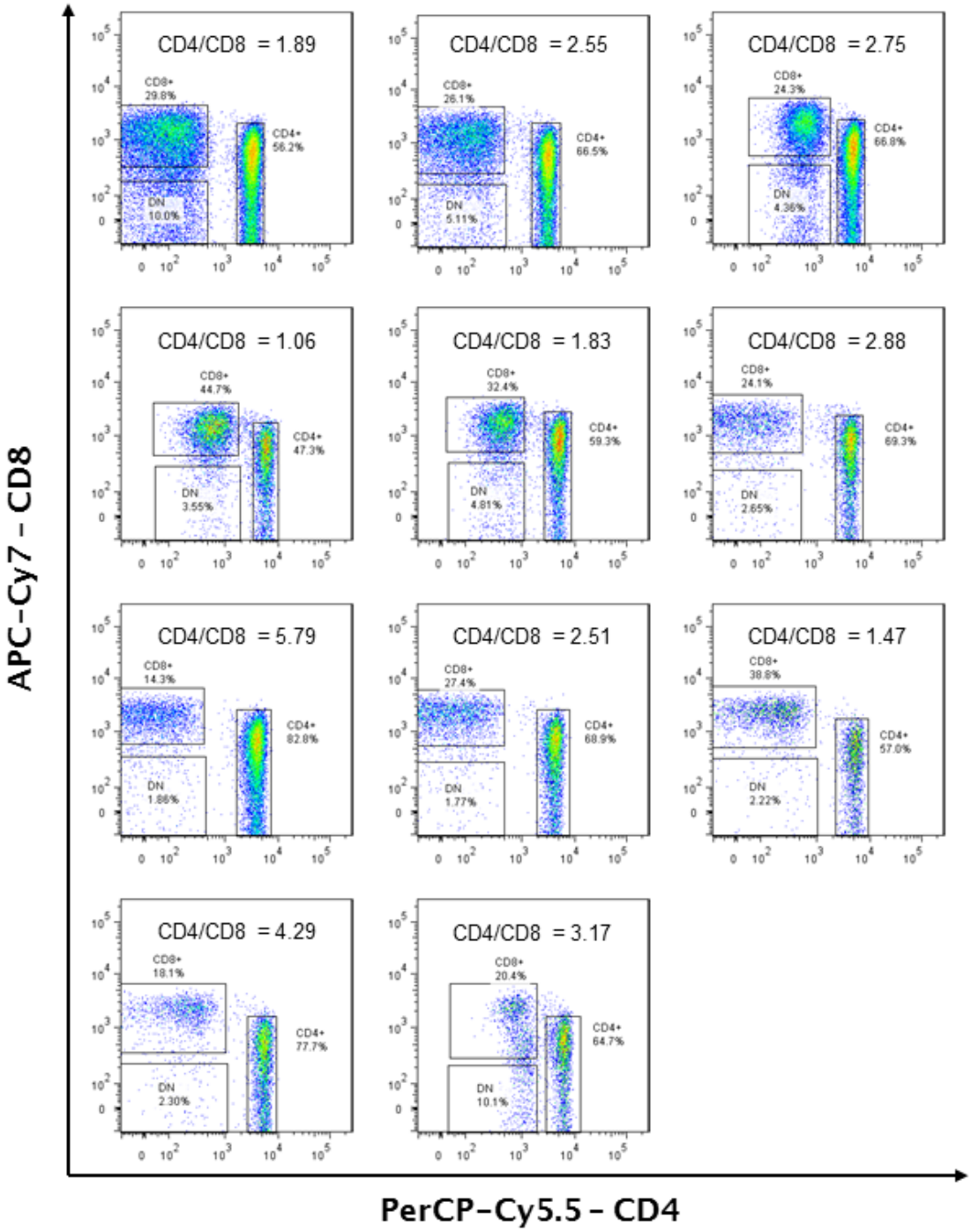
FLOW CYTOMETRY IMAGES

Figure 39: Results of Flow Cytometry Analysis of All Patients' PBMC - CD3+ T-Cells in Chronic Pancreatitis and Controls

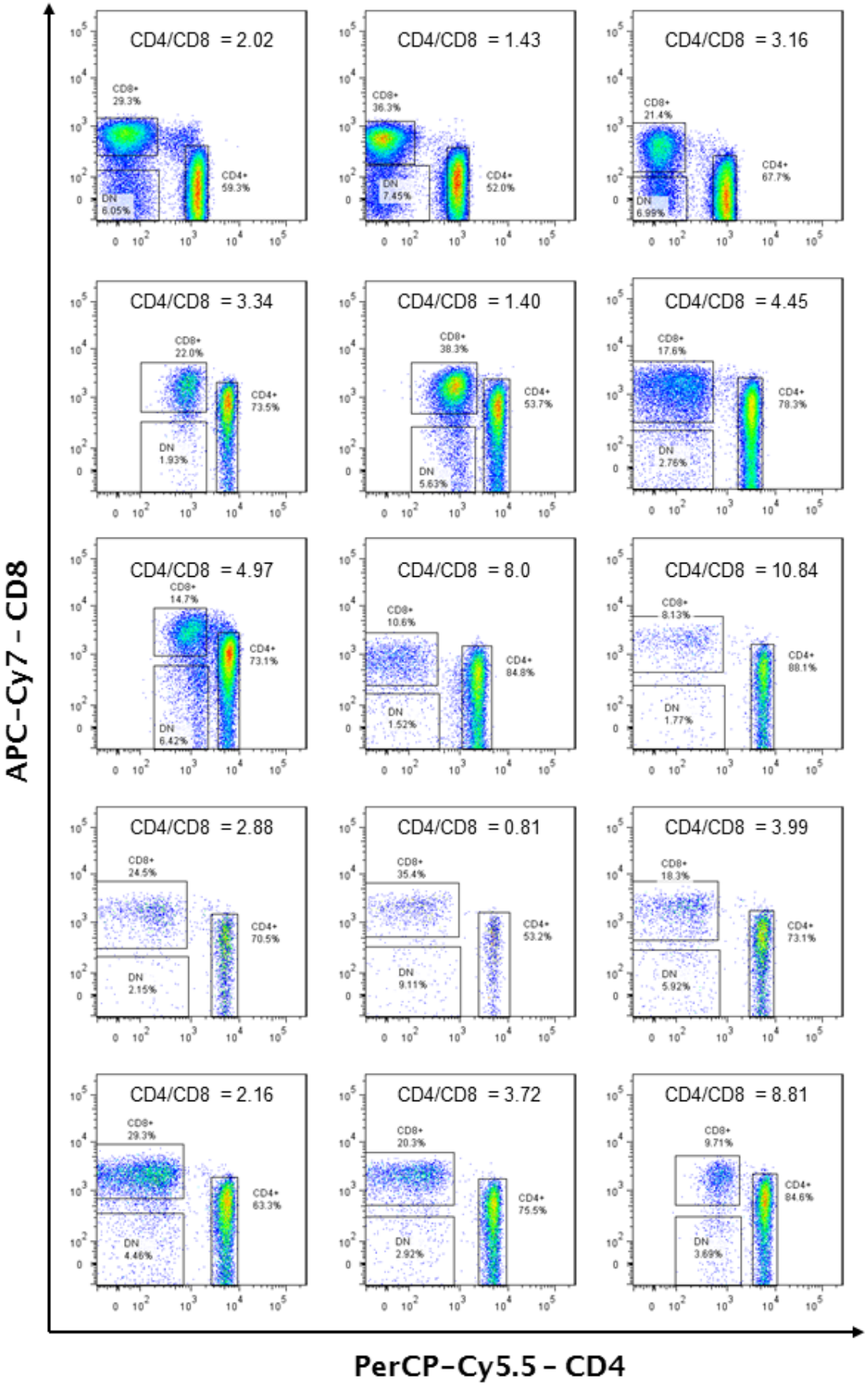
PBMCs from Patients with Chronic Pancreatitis - Gated on CD3+ T-Cells (1)



PBMCs from Patients with Chronic Pancreatitis - Gated on CD3+ T-Cells (2)



PBMCs from Normal Control Patients - Gated on CD3+ T-Cells (1)



PBMCs from Normal Control Patients - Gated on CD3+ T-Cells (2)

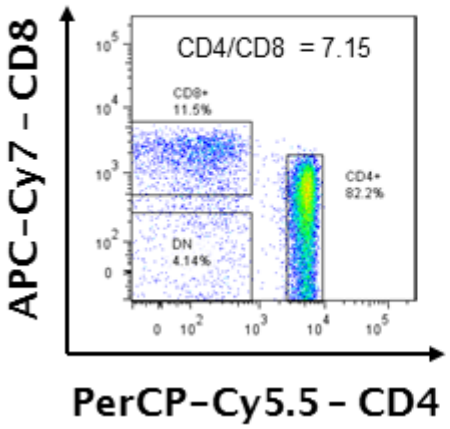
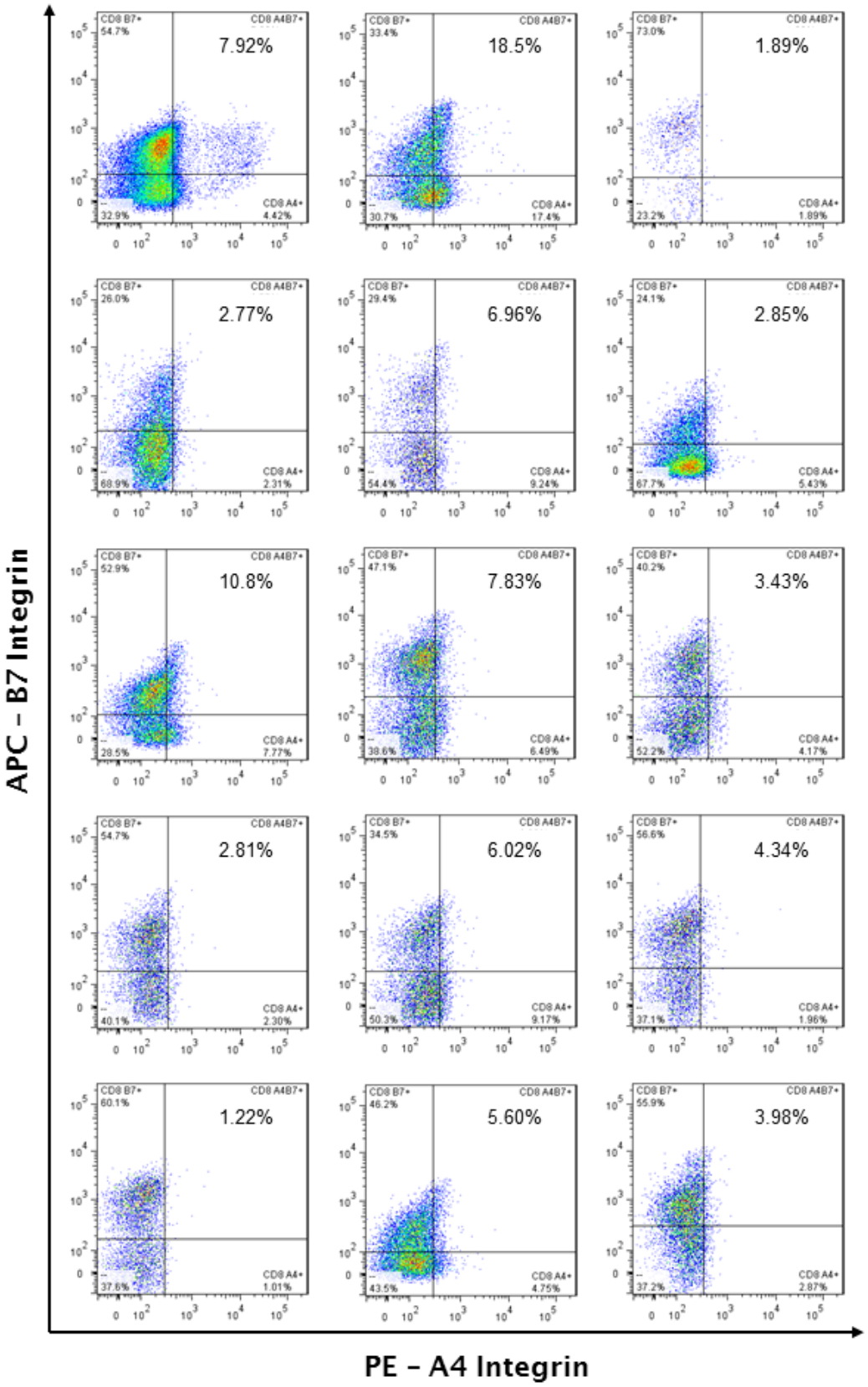
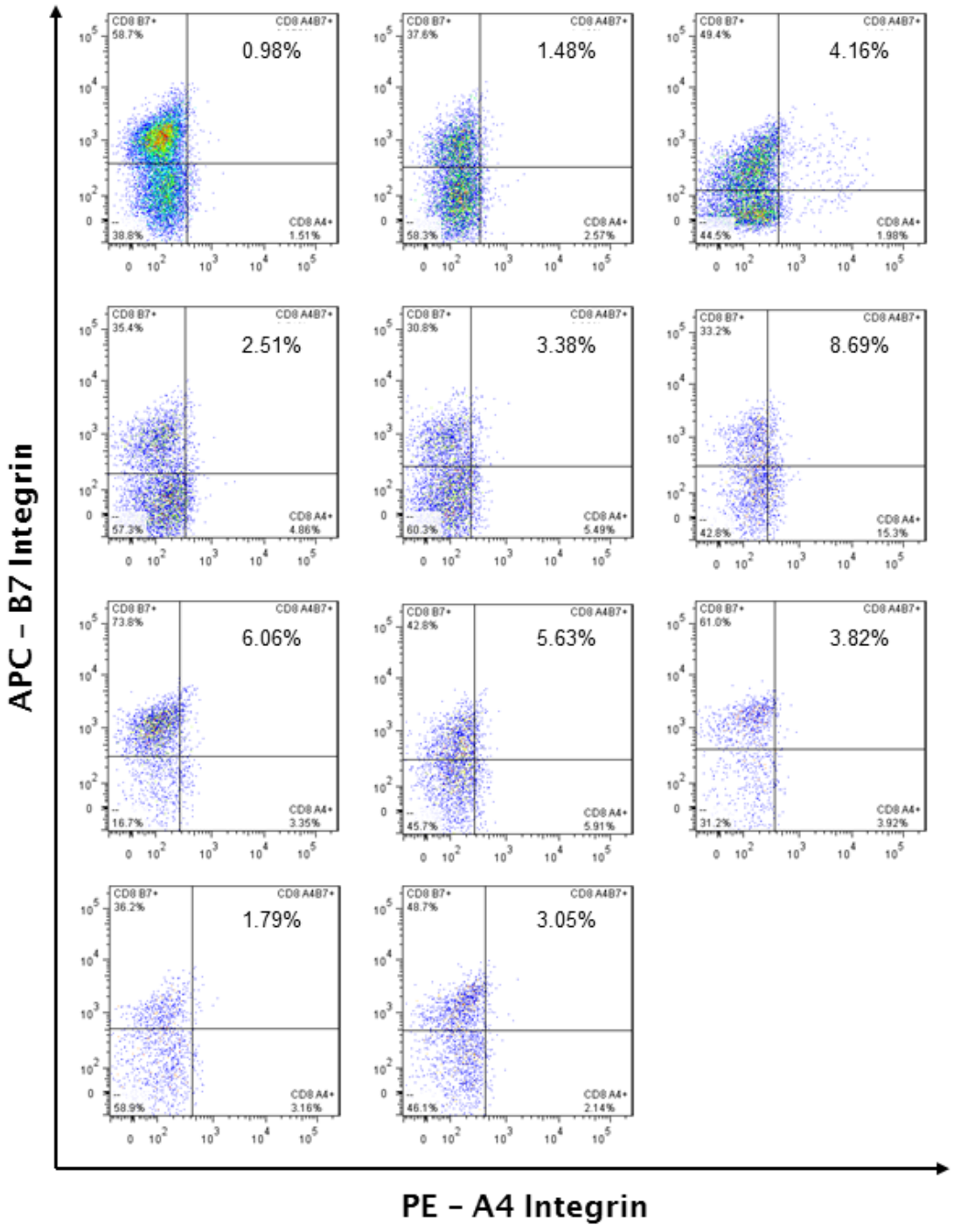


Figure 40: Results of Flow Cytometry Analysis of All Patients' PBMC - CD8+ Integrin α4+β7+ T-Cells in Chronic Pancreatitis and Controls

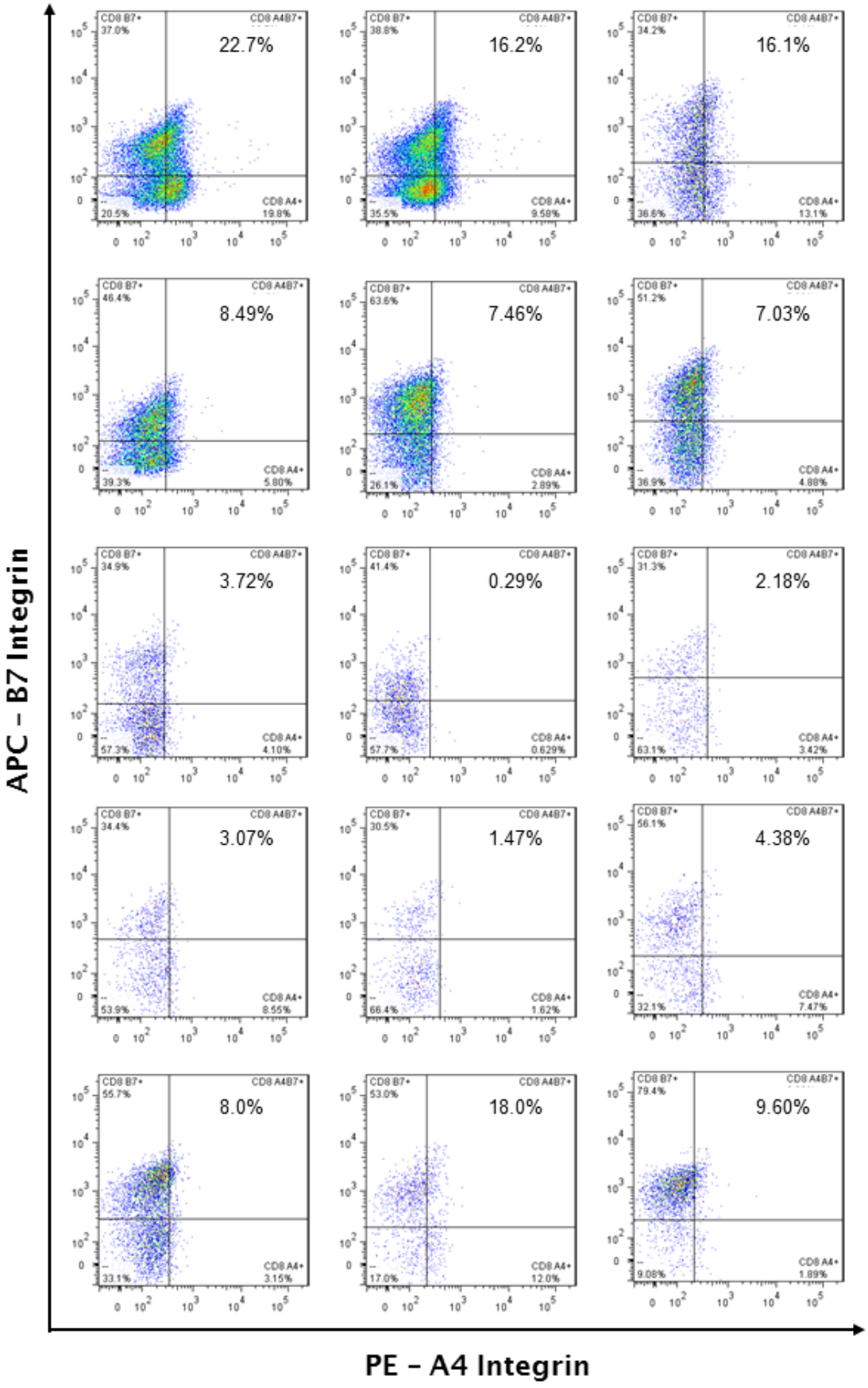
PBMCs from Patients with Chronic Pancreatitis - Gated on CD8+ T-Cells (1)



PBMCs from Patients with Chronic Pancreatitis - Gated on CD8+ T-Cells (2)



PBMCs from Normal Control Patients - Gated on CD8+ T-Cells (1)



PBMCs from Normal Control Patients - Gated on CD8+ T-Cells (2)

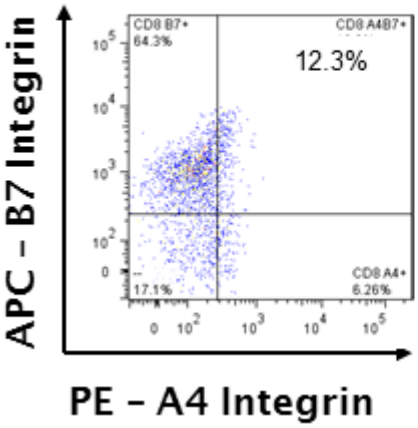
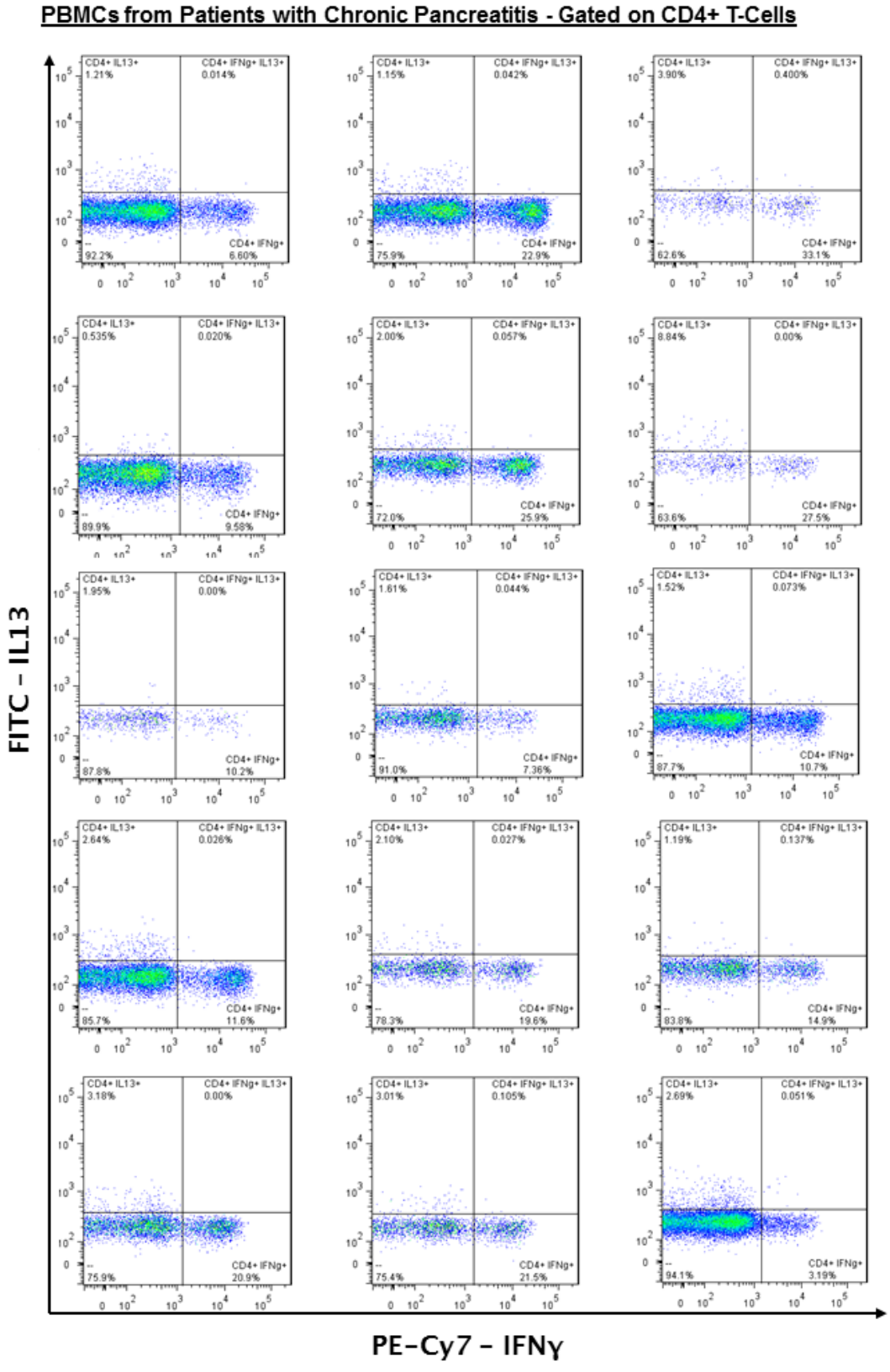


Figure 41: Results of All Patient's Peripheral Blood Flow Cytometry Analysis - Th1 and Th2 Cells in Chronic Pancreatitis and Controls



PBMCs from Control Patients - Gated on CD4+ T-Cells

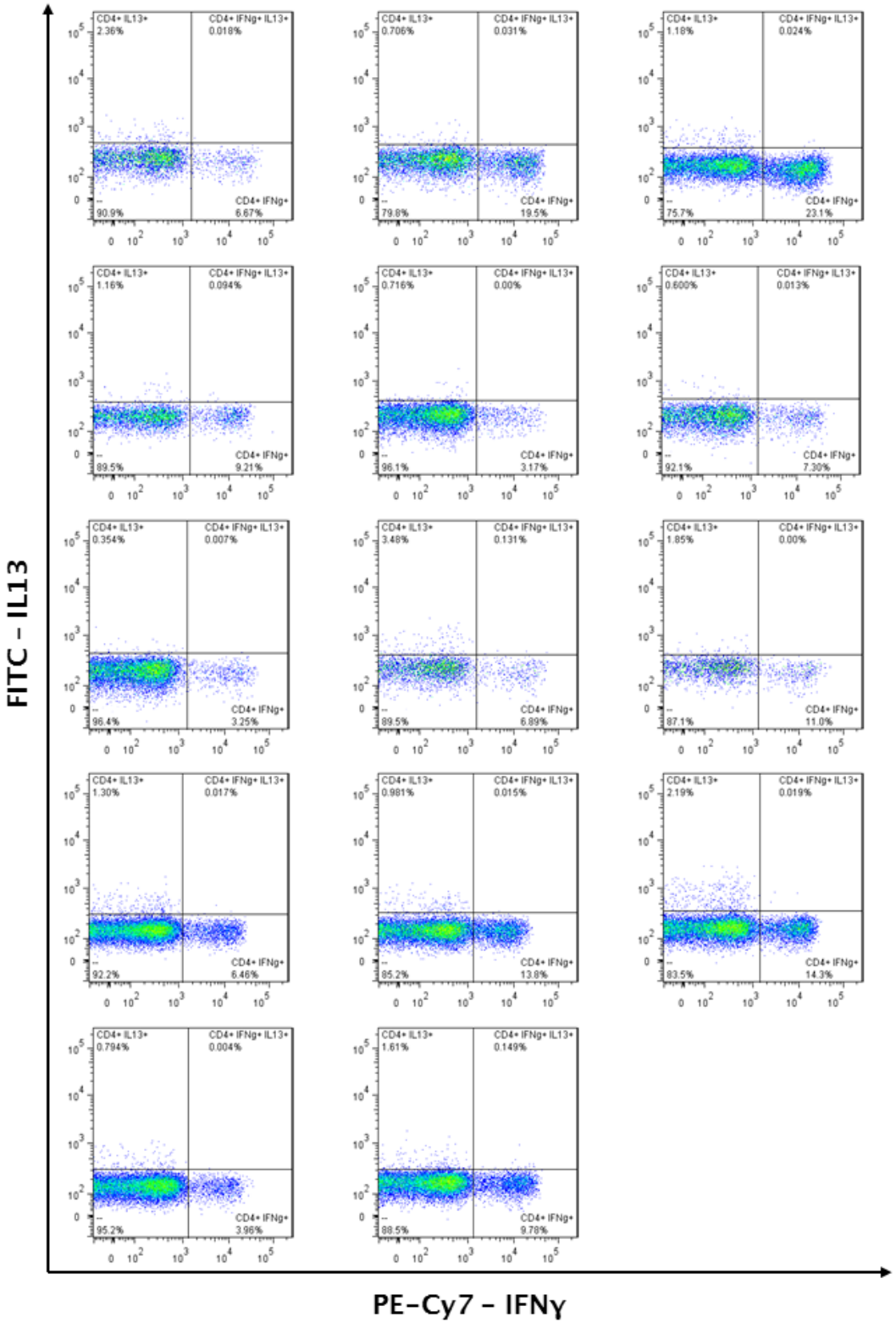
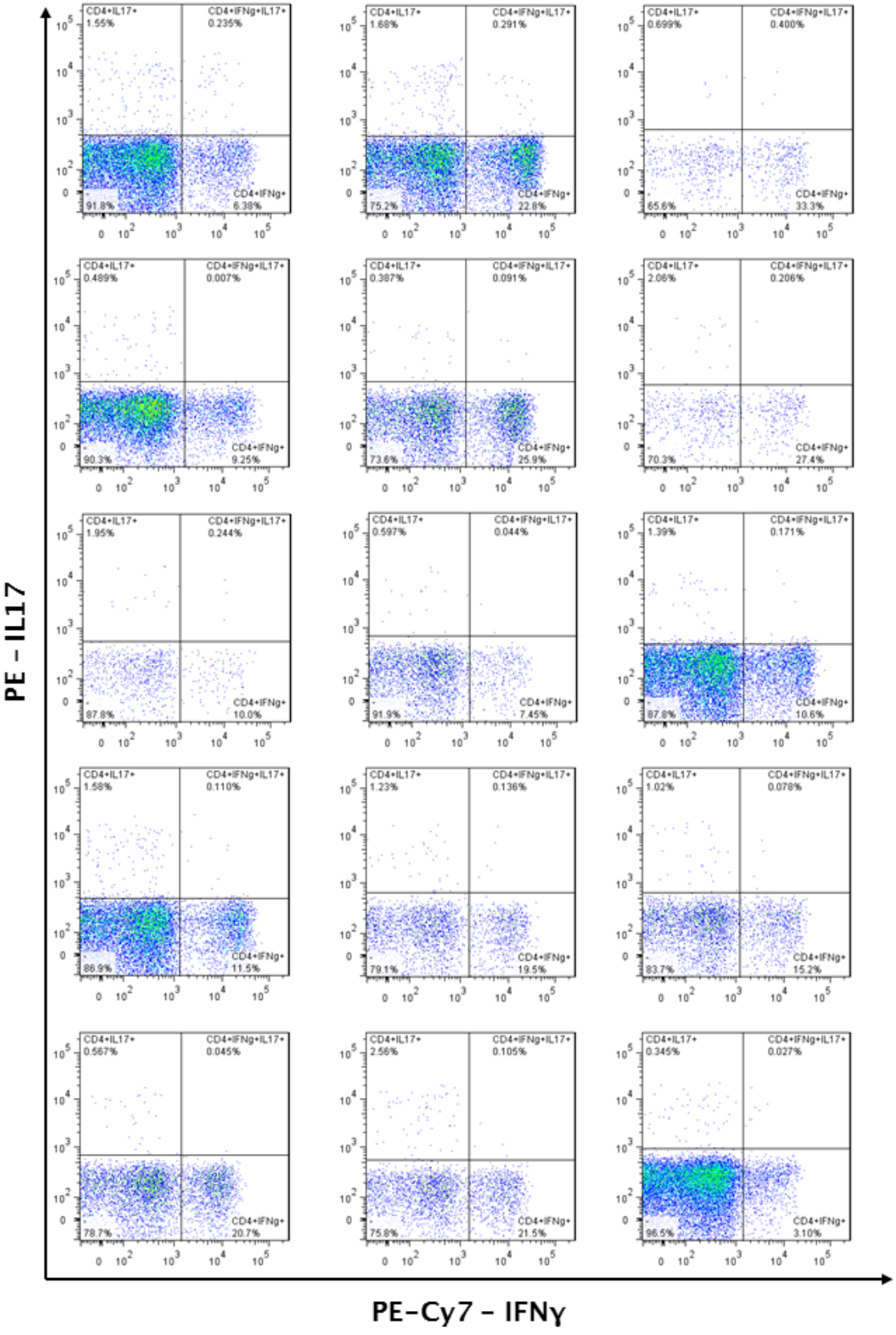


Figure 42: Results of All Patients' Peripheral Blood Flow Cytometry Analysis - Th1 and Th17 Cells in Chronic Pancreatitis and Controls

PBMCs from Patients with Chronic Pancreatitis - Gated on CD4+ T-Cells



PBMCs from Control Patients - Gated on CD4+ T-Cells

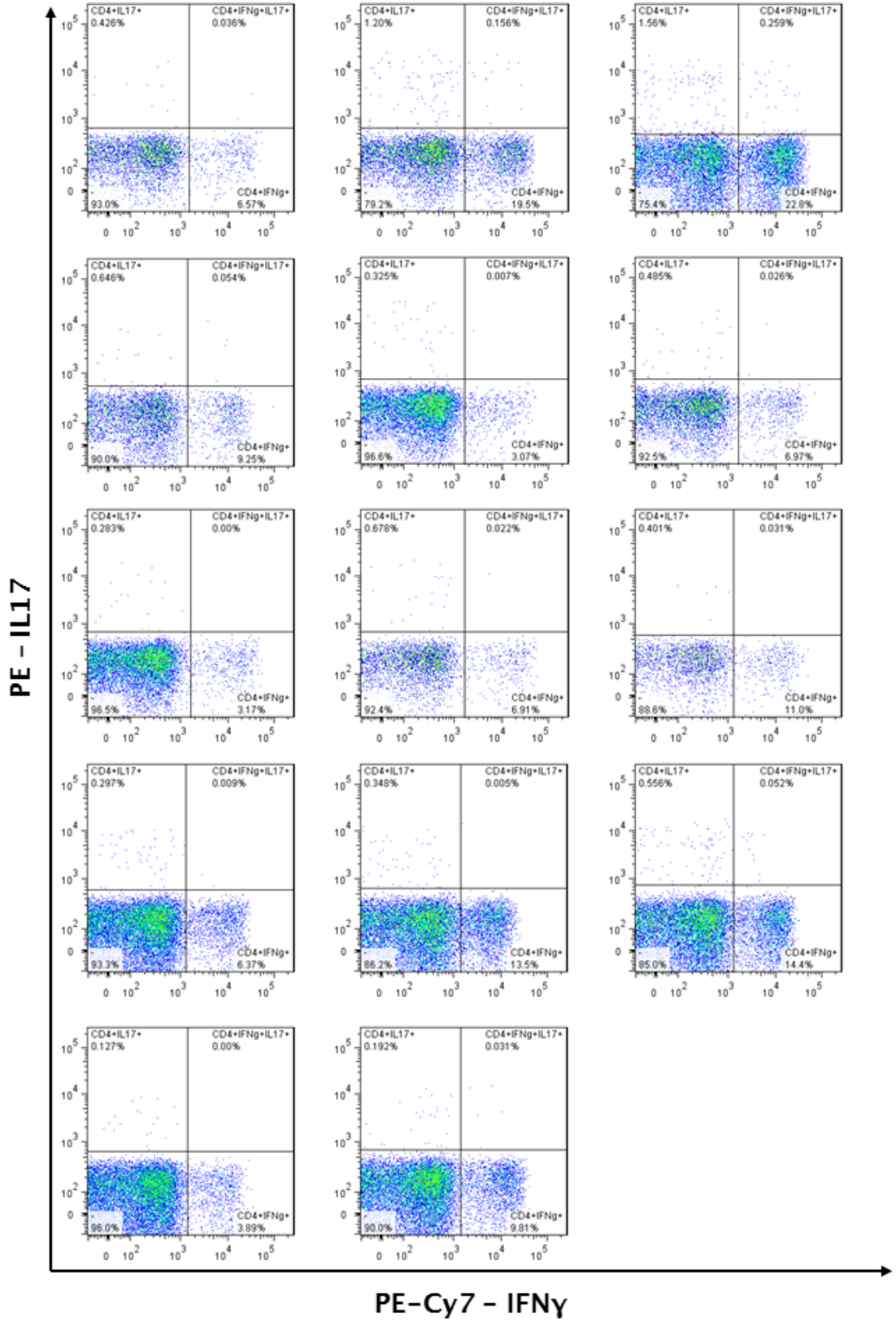
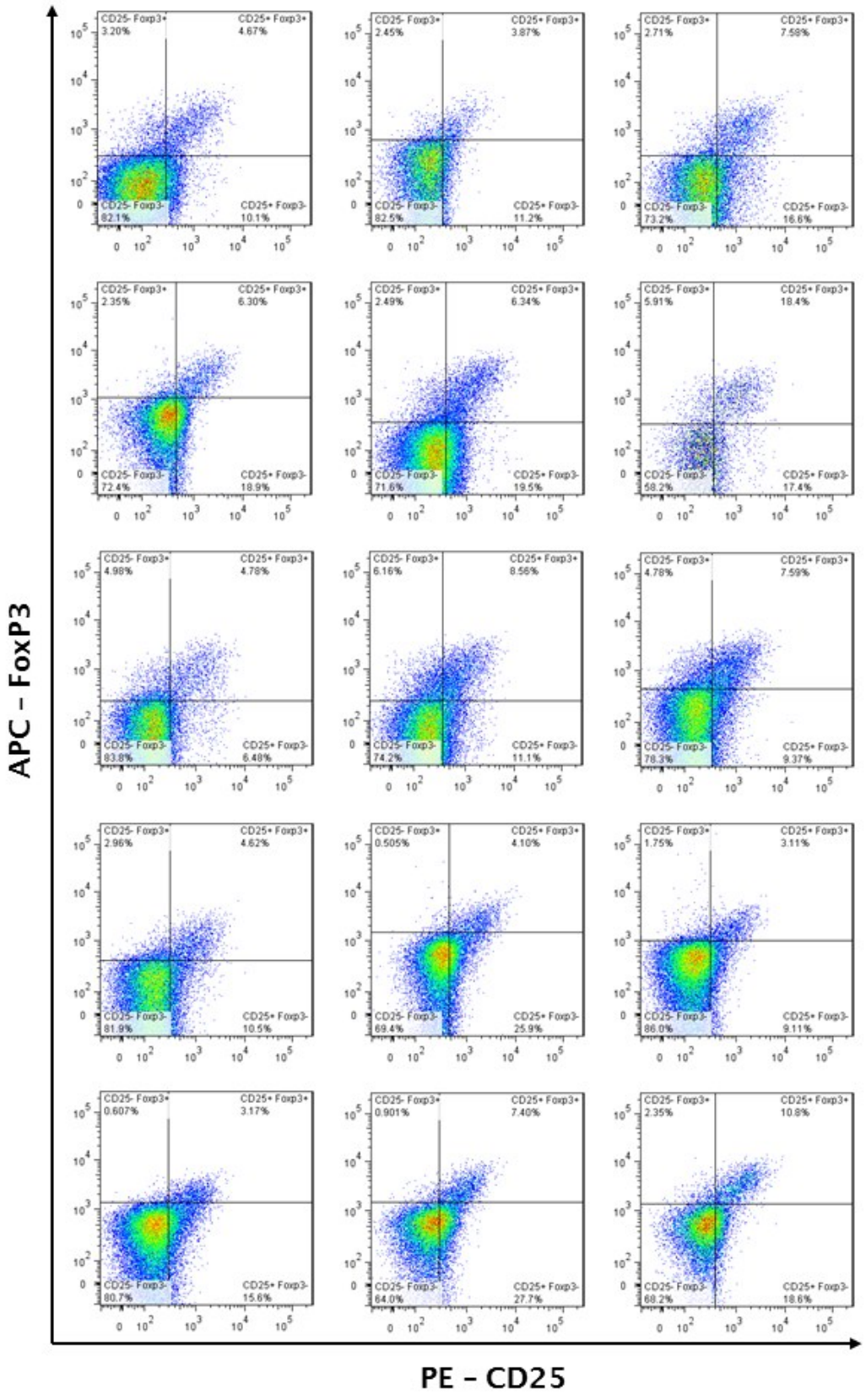
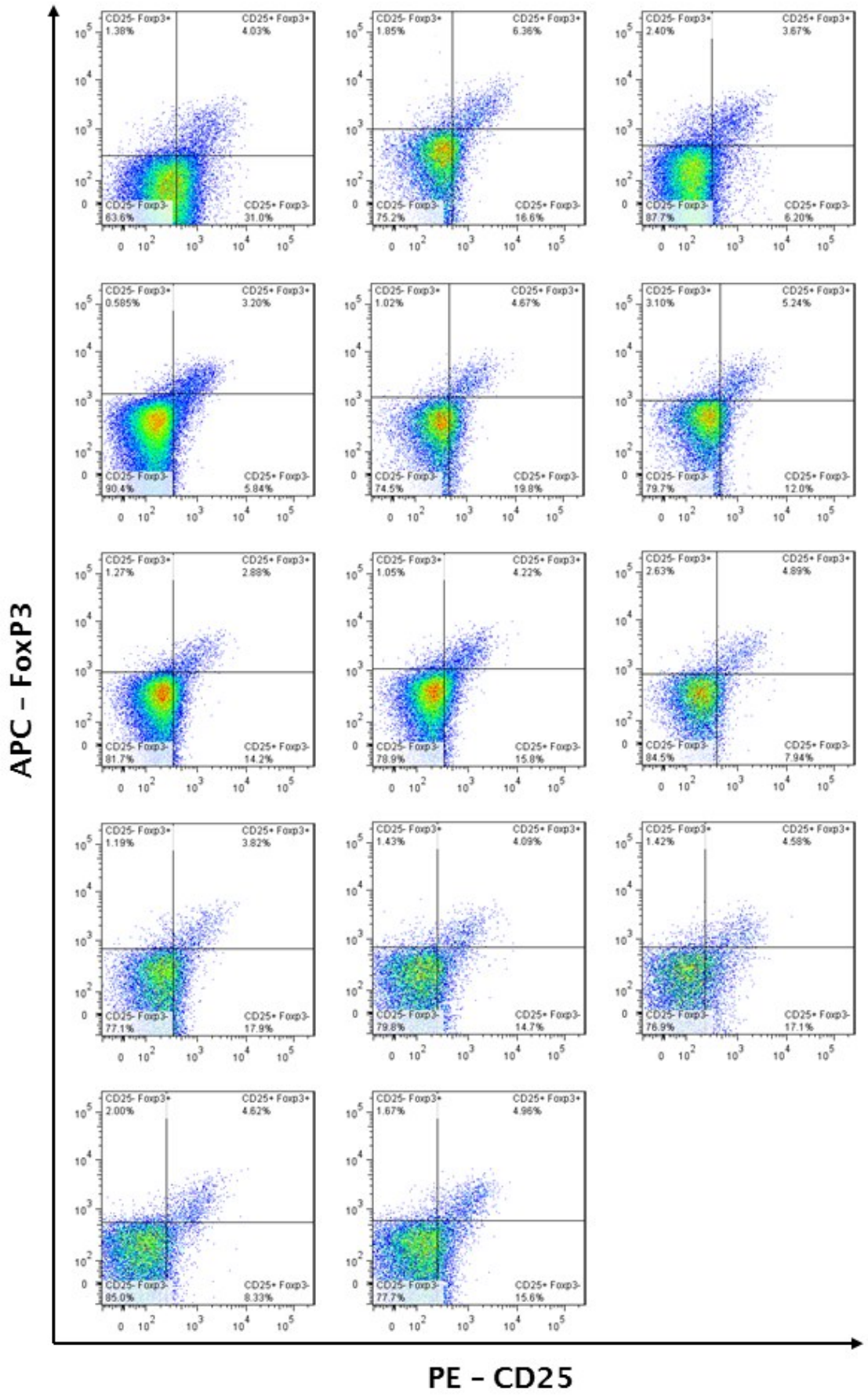


Figure 43: Results of All Patients' Peripheral Blood Flow Cytometry Analysis – T-Regulatory Cells in Chronic Pancreatitis and Controls



PBMCs from Control Patients - Gated on CD4+ T-Cells



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