Cloning and characterization of IA-2 specific autoantibodies in Type 1 Diabetes

Johnson, Carolyn Cromien

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King's College London

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Cloning and characterization of IA-2 specific autoantibodies in Type 1 Diabetes

A thesis submitted to Kings’ College London for the candidature of Doctor of Philosophy

Carolyn Johnson (Richardson)

March 2016

Division of Diabetes and Nutritional Sciences,
School of Medicine,
Kings’ College London
Abstract:

The presence of one or more autoantibodies to characterized autoantigens (GAD, IA-2, (pro) insulin, Znt8) is commonly used to diagnose or predict Type 1 diabetes. 94% of patients express antibodies to at least one of the major islet autoantigens at the point of diagnosis: of these 70% have autoantibodies to IA-2, and 94% of patients can be identified by screening for multiple autoantibodies. Autoantibodies to IA-2 are known to appear in early or pre-diabetes, suggesting that the appearance of B cell reactivity to this antigen is an early event in the progression to disease.

Recent studies have shown that Rituximab, a CD20 antibody, which depletes B cells, is able to preserve β-cell function in newly diagnosed patients, suggesting that B cell depletion may be an effective method of preventing β-cell destruction. Autoantigen specific B cell depletion in the NOD mouse, using a monoclonal anti-insulin antibody, restores immune tolerance to insulin. Similar strategies aimed at the specific depletion of islet autoantigen-reactive B cells may be effective in preventing diabetes progression in man. Development of such strategies requires knowledge of the nature of epitopes recognized by the B cell.

The purpose of this study was to characterize specific targets of autoimmunity in type 1 diabetes using human monoclonal antibody fragments, developed from B cell lines and antigen specific B cells, alongside B cells within the inflammation in the type 1 diabetic pancreas. Antibody binding of sites on the IA-2 antigen was evaluated using NMR. Finally, site directed mutagenesis was used to determine specific residues important for antibody binding within known epitope regions.

This study demonstrates the feasibility of generating monoclonal antibody fragments from monoclonal B cell lines, single B cells and archival material. This study also clarifies key regions required for autoantibody recognition of the juxtamembrane domain of the IA-2 protein, and provides a basis for future analysis of antibody binding of this antigen using NMR.
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Statement of originality

The work described in this thesis was carried out in the Division of Diabetes and Nutritional Sciences, King’s College London. Unless stated, the experiments were carried out by the author. The thesis entitled ‘Cloning and characterization of IA-2 specific autoantibodies in Type 1 Diabetes’ has not been submitted for a degree or other qualification at another university.

Carolyn Johnson

March 2016.
Publications arising from this thesis

Journal Publications:


Conference Abstracts:

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<td>Antibody</td>
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</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DPT-1</td>
<td>Diabetes prevention trial 1</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen binding domain of immunoglobulin molecule</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IA-2</td>
<td>Insulinoma associated protein -2</td>
</tr>
<tr>
<td>IAA</td>
<td>Insulin autoantibodies</td>
</tr>
<tr>
<td>ICA</td>
<td>Islet cell autoantibody</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JM</td>
<td>Juxtamembrane</td>
</tr>
<tr>
<td>LADA</td>
<td>Latent autoimmune diabetes in the adult</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MODY</td>
<td>Mature onset diabetes of the young</td>
</tr>
<tr>
<td>NOD</td>
<td>Non obese diabetic</td>
</tr>
<tr>
<td>PAS</td>
<td>Protein A sepharose</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RBA</td>
<td>Radiobinding assay</td>
</tr>
</tbody>
</table>
TBS  Tris buffered saline
TH1/2  T helper cell type ½
ZnT8  Zinc transporter 8
Chapter 1  Introduction

1.1  Introduction to diabetes:

The term diabetes mellitus covers several different metabolic diseases, which interfere with glycemic control. The most common is type 2 diabetes (usually non-insulin dependent), which is associated with greater age at onset and characterized by loss of glycemic control and worsening β-cell function over time. Other forms of diabetes include MODY (maturity onset diabetes of the young) and LADA (Latent autoimmune diabetes in the adult). MODY is a non-insulin dependent disease, usually associated with young age at onset, and autosomal dominant inheritance of genetic defects (Velho and Froguel 1998). LADA patients are traditionally defined by slow progression of the disease and antibodies to islet autoantigens (Pozzilli and Di Mario 2001).

Type 1 diabetes is a disease which occurs as a result of β-cell malfunction as a consequence of autoimmunity. Patients with type 1 diabetes are commonly diagnosed at puberty, with an enhanced risk conferred by the presence of first degree relatives with the disease (Lorenzen, Pociot et al. 1994). Unlike for other autoimmune diseases, both males and females are at an approximately equal risk of developing type 1 diabetes. Type 1 diabetes typically manifests itself in an insufficient uptake of glucose from the blood, leading to hyperglycemia, polyuria, polydipsia, weight loss and tiredness, resulting from T cell mediated autoimmune destruction of pancreatic β-cells of the islets of Langerhans, leading to insulin insufficiency (Eisenbarth 1986). Until the discovery of insulin by Banting and Best in 1922 (Banting, Best et al. 1922), the disease was fatal. Administration of exogenous insulin and islet transplantation are, to date, the only treatments available. Transplantation requires immunosuppression to prevent graft rejection, and exogenous insulin cannot fully compensate for the lack of endogenous insulin secretion, leaving patients at risk of development of secondary complications, including nephropathy, neuropathy and retinopathy.

Whilst only 5% of the diabetic population has type 1 diabetes, the rate of disease occurrence is increasing by 3% per year in the UK. Risks are conferred by a particular genetic background (HLA-DR3/4, DQ2/8), but a high risk genotype is not sufficient for disease to develop. Both environmental factors and genetic predisposition are necessary to induce disease.

The disease is closely associated with both B and T cell immunity to several β-cell antigens. The presence of antibodies to islet autoantigens are markers of autoimmune destruction of β-cells that occurs prior to and during disease onset (Gorsuch, Spencer et al. 1981; Ziegler, Hummel et al. 1999; Atkinson and Eisenbarth 2001). Autoantibodies specific to islet proteins (IA-2, IA-2β, GAD, insulin and Znt8 (Wenzlau, Juhl et al. 2007) are the best characterized, however there are many other less
characterized antigens (Glutamate transporter 1, PDX 1, Inwardly rectifying Potassium channels, Tetrastatin 7, NPY, Imogen 38 (as a bystander rather than target antigen)) (Taplin and Barker 2008; Zhang, Nakayama et al. 2008; Perego 2012; Wenzlau and Hutton 2013; McLaughlin 2015). Autoantibodies to the major islet antigens are known to occur prior to clinical disease onset. They may appear months or even years before disease symptoms (Gorsuch, Spencer et al. 1981; Ziegler and Nepom 2010). Autoantibody secreting B cells have often been considered to be secondary to T cell immunity in Type 1 diabetes, rather than a cause; due to the discovery that adoptive transfer of T cells from diabetic mice is capable of inducing disease in recipient mice (Christianson, Shultz et al. 1993). In more recent studies, both animal and human studies indicate a more central role for these cells in antigen presentation (Pescovitz, Greenbaum et al. 2009; Marino, Silveira et al. 2011). Therefore, a clarification of the role played by these immune cells in disease pathogenesis is necessary.

1.2 The pancreas in Type 1 diabetes

Type 1 diabetes is an autoimmune disease which specifically targets the endocrine β-cells of the pancreas, although there is some infiltration of the exocrine tissue, and minor exocrine atrophy (Rodriguez-Calvo, Ekwall et al. 2014). The pancreas itself is an unencapsulated, retroperitoneal organ, located just above the spleen. The organ is divided into four regions: head, body, tail and uncinate process as shown in Figure 1-1. The bulk of the pancreas consists of exocrine tissue including acinar cells, pyramidal cells clustered around ducts. The acinar cells secrete digestive enzymes (Trypsinogens, Chymotrypsinogen, Procarboxypeptidase A, Procarboxypeptidase B, Prophospholipase, Proelastase, and Mesotrypsin), ions and water into the duodenum. Other important cell types in the exocrine pancreas include duct cells, which are rich in mitochondria, required for ion transport between cells (Williams 2001).
Centroacinar cells are found at the junction of duct and acinar tissue, and are similar in structure to the duct cells, but derive from different progenitor tissue. Both ductal and centroacinar cells secrete bicarbonate (Steward, Ishiguro et al. 2005).

The pancreatic stellate cell, as the name suggests is a star-shaped cell, which is found wrapped around ductal, acinar and islet tissue. This cell type is most likely involved in laying down the basement membrane for the pancreatic epithelium (Omary, Lugea et al. 2007).

1.2.1 The Pancreatic Islet

Figure 1-2: Fluorescent confocal image of a non diabetic adult human pancreas. Labelling performed by myself with A. showing anti somatostatin (red) and anti glucagon (green) B. Fluorescence image showing control adult human pancreas labeled with anti insulin (green) and anti pancreatic polypeptide (red).
The islets of Langerhans are distributed throughout the pancreas, contributing approximately 2% of the total pancreatic mass. The islet is composed of five major hormone secreting endocrine cell types:

- **β-cells**, which secrete insulin in response to increased levels of blood glucose.

- **α-cells** which secrete glucagon in response to lowered blood glucose.

- **δ-cells** which secrete somatostatin, an inhibitor of growth.

- **ɛ-cells** which secrete ghrelin, a regulator of energy use and appetite (Andralojc, Mercalli et al. 2009).

- PP-cells secrete pancreatic polypeptide, which has several putative functions, including appetite regulation (Batterham, Le Roux et al. 2003).

As seen in Figure 1-2, somatostatin, glucagon and PP containing cells are distributed at the periphery of the human islet, whilst insulin forms a core group of cells. β-cells are the most abundant endocrine cell within the islet. The pancreas derives from the gut endoderm, and the initial signaling events in pancreas differentiation involve down-regulation of sonic hedgehog, and up-regulation of PDX-1. The endocrine cells of the pancreas derive from cells expressing Neurogenin-3 and Neuro-D.

During the neonatal period, endocrine cells undergo rapid maturation, via apoptosis, and begin to express the glucose sensing machinery of the mature β-cell (Richardson, Hussain et al. 2007). Mature islets are maintained by regular neogenesis and apoptosis (Finegood, Scaglia et al. 1995) permitting adjustment to differing demands for insulin secretion, during growth or pregnancy.

In Type 1 diabetes, it is predominantly the β-cells that are destroyed within the islet: this destruction is caused by a failure of immune tolerance, leading to immune recognition of β-cell antigens. Figure 1-3 shows the progressive loss of the insulin containing core of the islet, with A and D. showing control pancreas, B. and C. a recent onset diabetic sample and E. an established diabetic sample.
Figure 1-3: Immunohistochemical staining of human pancreatic islets. A. Insulin containing β-cells forming a core to the islet, becoming weaker in recent onset type 1 diabetic pancreas, with clear peri islet infiltration in a recent onset diabetic B. Glucagon stained alpha cells dispersed throughout the islet in control sections C. becoming more compacted in recent onset type 1 diabetic pancreas D. and forming the islet core in an established type 1 diabetic sample E. Labeling performed by myself using sections of pancreas provided by Dr Anne Clark, OCDEM, University of Oxford

A less well observed feature of type 1 diabetes is exocrine atrophy (Frier, Saunders et al. 1976): this combined with the rare occurrence of anti-pancreatic cytokeratin and bile salt-dependent lipase antibodies in type 1 diabetic individuals (Drell and Notkins 1987), suggest that the immune response is not solely directed at the pancreatic β-cell.
1.2.2 Pathogenesis of Type 1 diabetes

![Schematic of the pathogenesis of type 1 diabetes](image)

Figure 1-4: Schematic of the pathogenesis of type 1 diabetes. Showing initiation of disease involving infiltration of the islet by APCs (monocytes, NK cells, macrophages, dendritic cells and B cells) into the pancreatic islets. APCs move out to peripheral lymph nodes and present β-cell antigens to T cells, leading to T cell activation and progressive destruction of the islet β-cells, culminating in disease.

Type 1 diabetes is an autoimmune disease, initiated by an inappropriate immune response to pancreatic islet autoantigens: a bone marrow transplant between HLA matched siblings is sufficient to transfer type 1 diabetes between individuals (Lampeter, Homberg et al. 1993), demonstrating that the immune system is the cause of β-cell destruction. At the time of disease onset, islets are infiltrated by immune cells, as shown in the schematic in Figure 1-4. Intra and peri islet B cell infiltration in a recent onset type 1 diabetic sample can be seen in Figure 1-5. Not all islets are infiltrated, and whole lobes of the pancreas can be seen to be spared at disease onset (In't Veld 2014).
Figure 1-5: Section of pancreas from a recent onset type 1 diabetic patient. Showing B cells (stained brown with anti-CD20) infiltrating the islet.

Insulitis was first described in 1902, by Schmidt, in a ten year old patient who had died of diabetic ketoacidosis (Schmidt 1902). The infiltration was then named insulitis by Von Meyenburg (Meyenburg. 1940). The most recent definition of insulitis, which is described as an immune cell infiltration of the islets of Langerhans, is shown below in Table 1-1 adapted from Campbell Thompson et al (Campbell-Thompson, Atkinson et al. 2013).

Table 1-1: JDRF-NPOD consensus definition of insulitis

<table>
<thead>
<tr>
<th>Consensus definition of insulitis (Campbell-Thompson, Atkinson et al. 2013)</th>
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<tbody>
<tr>
<td>Patients with insulitis are defined by the presence of a predominantly lymphocytic infiltration specifically targeting the islets of Langerhans. The infiltrating cells may be found in the islet periphery (peri-insulitis), often showing a characteristic tight focal aggregation at one pole of the islet that is in direct contact with the peripheral islet cells. The infiltrate may also be diffuse and present throughout the islet parenchyma (intra-insulitis). The lesion mainly affects islets containing insulin-positive cells and is always accompanied by the presence of (pseudo)atrophic islets devoid of β-cells. The fraction of infiltrated islets is generally low (&lt;10% of islet profiles). The lesion should be established in a minimum of three islets, with a threshold level of $\geq 15$ CD45+ cells/islet before the diagnosis can be made. The pathology report should include the total number of islets analyzed, the fraction of islets affected by insulitis, the fraction of (pseudo)atrophic islets, and a description of the spatial relationship of the infiltrate to the insulin-positive islet cells.</td>
</tr>
</tbody>
</table>
Using the definition generated by the JDRF-nPOD working group (Campbell-Thompson, Atkinson et al. 2013), it has been determined that insulitis generally occurs in less than 10% of islets. The group also determined that insulitis can be either restricted to the periphery of the islet, (peri-insulitis), or a more dispersed infiltration, occurring throughout the islet, (intra-insulitis). Active infiltration of the endocrine pancreas can be seen in Figure 1-6, with dense lymphocytes exiting a capillary, and infiltrating the endocrine pancreas. Insulitis occurs in islets containing β-cells, and is usually accompanied by atrophied islets with no remaining β-cells in the same lobe (Gepts 1965). The infiltrate consists of a majority of CD8+ T cells, with some CD4+ lymphocytes, B cells and macrophages (Willcox, Richardson et al. 2009). This cellular infiltration of the islet occurs around the same time as detectable humoral immunity, when autoreactive T and B cells can be found in circulation. The first strong evidence for islet autoimmunity was reported in 1963, when Pav revealed the presence of insulin autoantibodies in type 1 diabetic patients (Pav, Jezkova et al. 1963) this was followed up 1974, when Bottazzo et al observed anti-islet cell antibody reactivity in sera taken from patients with type 1 diabetes and patients with other endocrine autoimmune disorders (Bottazzo, Florin-Christensen et al. 1974). This was followed by the discovery in 1983, of the presence of insulin antibodies in type 1 diabetic patients, prior to treatment with exogenous insulin (Palmer, Asplin et al. 1983).

Figure 1-6: Electron micrograph of pancreatic islet. Showing lymphocytes (L) exiting capillary (C) and infiltrating endocrine pancreas (courtesy of Dr. A. Clark).
Type 1 diabetes was believed to be a T cell mediated disease (Roep 1996): T cells are considered to play a central role in the destruction of β-cells, that ultimately leads to insulin deficiency and a lifelong dependency on exogenous insulin. Pathogenesis begins years before clinical disease onset: Regulatory mechanisms are believed to prevent the actions of autoreactive effector T cells prior to disease onset: these regulatory elements may include regulatory T cells (CD4+ Tregs) and possibly invariant NKT cells (Diana, Beaudoin et al. 2011; Diana, Brezar et al. 2011). It has been proposed that as disease begins, B cells and APCs, load β-cell peptides onto class I and II MHC, and present these peptides to T cells. As disease progresses, regulatory elements fail, and infiltration of the pancreatic islets, taking the form of insulitis, continues. Autoimmune infiltration and destruction of the islets of Langerhans continues for years after disease onset, and eventually, after 2-3 years of insulin therapy, >90% of β-cells are destroyed, although it is possible that in some younger patients, some degree of β-cell regeneration may occur (Butler, Meier et al. 2007). Data from several recent studies suggest that 70% of islets are completely lacking in insulin, whilst only 1% of islets are infiltrated (Gregg, Moore et al. 2012). Although islets are β-cell deficient, they have normal numbers of other endocrine cell types, α-cells, γ-cells, δ-cells and PP cells. Infiltrating cells include CD8+ T cells, CD4+ T cells, macrophages, dendritic cells, B cells and plasma cells. Regulatory T cells and NK cells are rarely found in islet infiltrates.

Animal models of Type 1 diabetes, NOD (Non Obese Diabetic) mouse and the BB-DP (Bio Breeding diabetes Prone) (Bach 1994) rat both develop similar disease characteristics to humans: peri insulitis is followed by invasive intra islet insulitis. Pro-inflammatory IFN-γ secreting T cells invade the islet and autoantibodies to islet autoantigens can be detected (Carrero, Calderon et al. 2013).

1.3 The immune system in a healthy state

In healthy individuals, the immune system acts to defend the organism from infection. Defense from infection is mediated by a specialized group of immune cells, which express several specific receptors, capable of recognizing pathogenic microorganisms and viruses. The mechanisms by which cells recognize pathogens are specific to each immune cell type: B cell receptor (BCR), T cell receptor (TCR), CD molecules, and secreted molecules, including antibodies, chemokines, cytokines and complement molecules.

The cells of the immune system are divided into innate response cells (monocytes, macrophages, neutrophils and NK cells) and those forming the adaptive immune system (humoral B cells and T cells). Adaptive immunity is defined by the specificity of the response to the pathogen, and the ability to generate a ‘memory’ response, quickly activated should the same pathogen be encountered in the future. The specificity of this response is controlled by the particular bias of
immune cell receptors, TCRs or BCRs. Added to this, the secretion of antibody, having the same specificity as the B cell receptor, allows neutralization of circulating pathogens. The whole repertoire of different clones allows recognition of a huge variety of pathogens. The Burnet hypothesis (1959) of clonal selection portrays the system as one which selects clones for their ability to specifically bind antigen, and both expand and diversify those clones, in order to respond to pathogens and develop a memory response to them. In parallel to this positive selection, a system of negative selection exists to delete any clones which strongly recognize self-antigen: clonal deletion. A failure of clonal deletion leads to autoimmunity.

In the adult immune system, the average number of immune cells could be $7 \times 10^9$ cells per liter (Revillard 2001). Of these, ~30% are lymphocytes, and the majority of these are T cells (~75%) with B cells forming the minority (~10% of total lymphocytes); absolute values are shown in Table 1-2.
Table 1-2: Lymphocyte values in the blood of a healthy adult, adapted from Revillard (Revillard 2001)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percentage (range)</th>
<th>Absolute Numbers (10^9 cells/l) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+ B cells</td>
<td>11.8 (5-25)</td>
<td>0.2 (0.06-5)</td>
</tr>
<tr>
<td>CD3+/CD4+ T cells</td>
<td>45 (30-61)</td>
<td>0.8 (0.5-1.6)</td>
</tr>
<tr>
<td>CD3+/CD8+ T cells</td>
<td>28 (13-43)</td>
<td>0.5 (0.2-1)</td>
</tr>
<tr>
<td>CD3-/CD16+/CD56+ NK cells</td>
<td>14.6 (3-36)</td>
<td>0.3 (0.05-0.7)</td>
</tr>
<tr>
<td>Total Lymphocytes</td>
<td></td>
<td>2.5 (1.5-4)</td>
</tr>
</tbody>
</table>

1.3.1 The T cell

Cytotoxic T cells express CD8 on the cell surface: these cells localize to the site of infections, and kill target cells. Type 1 CD8+ cells which produce interferon (IFN)-γ, and type 2 cells, produce interleukin (IL)-4, IL-5, and IL-10. Both types of cytotoxic cell kill via perforin and Fas mediated cell death pathways. The CD8 molecule interacts with MHC I. Recognition of antigen presented on MHC I leads to induction of target cell death by the cytotoxic T cell.

CD4+ helper T cells express the glycoprotein CD4 on the plasma membrane. These cells are labeled ‘helper’ cells as they can activate other immune cell types including B cells, macrophages, NK cells and cytotoxic T cells, by secreting cytokines. The CD4+ T cell is activated is activated by engagement with peptide derived from antigen bound to MHC class II molecules, expressed by antigen presenting cells, including macrophages, dendritic cells and B cells. These CD4+ cells can be subdivided into several broad types: TH1, TH2, TH17 and follicular T helper cells (Kara, Comerford et al. 2014).

Both CD4+ T helper cells and CD8+ cytotoxic T cells are derived from double positive CD4+/CD8+ cells in the thymus.

TH1 cells are activated in the presence of IL12. STAT4 upregulation of Tbet leads to production of IFN-γ although these cells can also produce IL21. At the site of inflammation, TH1 cells produce CXCL9, CXCL10 and CXCL11, whilst acting to induce activation of macrophages and antigen-specific cytotoxic T lymphocytes (CTLs).

TH2 cells are activated via the STAT6 pathway, leading to IL4 expression, and upregulation of GATA3. The cells produce IL4, IL5 and IL13. These cells express CCR3, CCR4 and CCR8. TH2 cells activate basophils, eosinophils, and promote antibody-dependent cell-mediated cytotoxicity (ADCC).
subset of TH2 cells, TH9 cells are distinguished by their requirement for IL-4 and TGF-β. These cells secrete CCR3, CCR6, and CXCR3.

TH17 cells require the presence of IL-6, and TGFβ1, induced by STAT3 activation which leads to expression of RORγt leading to induction of IL-17A and IL-17F. TH17 cells can produce IFN-γ, IL-4, or IL-13. A subset of TH17, TH22 cells require IL-6 and TNF-α. These cells produce IL-22 under the control of the aryl-hydrocarbon receptor (AhR). TH22 cells express chemokine receptors CCR4, CCR6, and CCR10.

Follicular helper T cells are specific helper T cells, located in the follicles of the lymph nodes acting to aid the maturation of clonal B cells. These cells express Bcl6, and cytokines IL-4, IFN-γ, IL-21, and IL-17A. These cells also express high levels of the chemokine receptor CXCR5 which controls migration into germinal centers. Kenefeck et al demonstrated that there is an increase of IL-21 producing follicular helper T cells in the lymph nodes of diabetic patients (Kenefeck, Wang et al. 2015), and that numbers of follicular helper T cells correlates with the presence of autoantibodies to IA-2 ad ZnT8, but not GAD65 (Kenefeck, Wang et al. 2015).

Memory T cells are long lived antigen specific T cells which persist in the periphery, to be reactivated upon antigen stimulation. Whilst more than 90% of effector cells generated die within the contraction phase, after clearance of antigen, the residual cells consist of long lived, quiescent memory cells, capable of proliferation in the absence of stimulation by peptide-MHCII (Seder and Ahmed 2003). There are two known classes of memory T effector memory T cells which encourage migration of cells the location of new inflammation (Reinhardt, Khoruts et al. 2001), generating effector cytokines, characteristically IFN-γ, IL-4 and IL-5. The central memory T cells proliferate, and secrete IL-2; central memory T cells also express CD62L and receptor CCR7, guiding T cell migration into the lymph node (Cyster 2005).

The T cell receptor (TCR) is expressed by all T cells. T cells in the peripheral immune system express αβ TCRs (γδ TCRs are most commonly found in the gut). Diversity of the TCR is generated by recombination, under the control of RAG proteins, as is also the case for immunoglobulin molecules (Roitt 1998). The TCR interacts with HLA to elicit a response to antigen. The T cell repertoire is controlled by thymic selection, which is a process designed to remove any T cells which generate a receptor with a high affinity for self-antigen. High affinity autoreactive TCRs are deleted, whilst low affinity autoreactive TCRs may escape into the periphery (Lohmann, Leslie et al. 1996). Thymic selection is primarily modulated by the thymic epithelium, which presents an appropriate range of self-epitopes to test reactivity of TCRs: in type 1 diabetes, a failure of negative selection, and
resultant deletion of autoreactive TCRs, is believed to be partly responsible for disease (Amrani, Verdaguer et al. 2000).

Any autoreactive T cells that have escaped into the periphery must be controlled. There are several mechanisms used by the immune system to reduce peripheral autoimmunity, including anergy, an unresponsive state of T cells induced by stimulation of peptide/MHC in the absence of co-stimulation (Maeda, Nishikawa et al. 2014). Autoreactive T cells may also be deleted in the periphery, when the cell is stimulated by an antigen presenting cell, expressing both MHCII and a death receptor, from the CD95 or TNFR family (Roitt 1998). Finally, cells can be controlled by regulatory T cells found in the periphery (Buckner and Ziegler 2004).

Regulatory T cells (Tregs) occur as natural Tregs, developing in the thymus, and adaptive Tregs (iTreg, Tconv) developing in the periphery. These cells are all typically CD4+/CD25+ with a wide range of TCRs (Takahashi, Kuniyasu et al. 1998) some of which may be autoreactive. iTregs, or Tconvs are generated in the periphery in an environment rich in regulatory cytokines. FOXP3 is believed to be expressed by the majority of Tregs and has been used as a marker for them (Bennett, Christie et al. 2001). However, mounting evidence suggests that FOXP3 is also expressed by cells of a pro inflammatory phenotype, expressing cytokines such as IFNγ and IL-17 (Corthay 2009). Both CD4+ and CD8+ T cells can be regulatory T cells (Maeda, Nishikawa et al. 2014). Unfortunately, identification of Tregs is complex, as so called ‘regulatory’ markers, including CD25, CTLA-4, GITR, LAG-3, CD127 and FOXP3 are now thought to be more general markers of T cell activation, not necessarily markers of a solely regulatory state (Corthay 2009).

Tolerance can be stimulated by expression of specific co-receptors on antigen presenting cells. Both CD80 and CD86, expressed by B cells and dendritic cells bind CD28 and CTLA-4, which is expressed by the T cell. Binding of CD28 enhances clonal deletion in the thymus, and promotes differentiation into a regulatory phenotype (Lenschow, Walunas et al. 1996).

1.3.2 The Role of B cells in the immune system:

B lymphocytes are important effector cells of the adaptive immune system. These cells are able to both produce and secrete immunoglobulin, which binds specific molecules on pathogens, immobilizing them and therefore targeting them for destruction. Immunoglobulin is also present as a membrane bound form on the B cell surface (the B cell Receptor: BCR), which can bind epitopes on potentially harmful molecules for subsequent internalization of the antigen. The B cell then digests the antigen, and presents peptides derived by antigen processing on MHC class II (HLA II) molecules on the cell surface. Presentation of epitopes in this format allows activation of CD4+ T cells.
B cells are derived from hematopoietic stem cells in the bone marrow. In order to become mature B cells they must undergo random rearrangement of both their heavy and light immunoglobulin chains, which takes place is several cycles, in both the central organs of the immune system and the periphery. This rearrangement, characterized by its sequential format, then determines the specificity of the antibody produced. Once a B cell has rearranged both heavy and light chain genes, further rearrangement is inhibited (Roitt 1998).

1.3.3 Immunoglobulin gene rearrangements in B cells:

![Figure 1-7: Rearrangement of the immunoglobulin heavy chain. Seen in A. and of the light chain in B. Within the B cell, one V, D and J gene are selected and brought together to form a functional V, D, J (or VJ for the light chain).]

The genes holding the information for human immunoglobulins are found on chromosome 14 (heavy chain), chromosome 2 (kappa light chain) and chromosome 22 (lambda light chain) (Cook and Tomlinson 1995; Matsuda, Ishii et al. 1998). Immunoglobulin genes are present in the genome as multiple gene segments, which must be rearranged within a B cell to form a functional gene. There are four sets of genes for the heavy chain: these are Variable, Diversity, Junctional, and Constant regions (shown in Figure 1-7). In humans, these consist of 51 V genes, 25 D genes and 6 J genes. One gene from each V, D and J are selected and brought together along with a constant gene to enable transcription and translation of antibody within the B cell. For the light chain, only V and J genes are used, but there are two types of light chain, kappa and lambda. For the kappa chain, one of 40 V genes and one of 5 J genes is selected and brought together along with a constant chain to enable transcription into mRNA. Lambda light chains have 31 possible V genes and 5 possible J genes, as shown in Figure 2. This rearrangement permits variation in the complementarity determining
regions (CDR), allowing binding of many different antigens (Roitt 1998). It also presents a challenge in cloning and sequencing of recombinant antibodies: only mRNA within the cell is representative of the antibody it produces, and antibody sequences from different B cell clones will not be the same. This means a broad range of primers must be used to ensure the ability to amplify all possible combinations of variable genes expressed, usually accomplished by use of a cocktail of degenerate primers, with careful selection of annealing temperature (Tiller, Busse et al. 2009).

The expression of Immunoglobulin genes begins with expression of *Rag1* and *Rag2* genes in the B-lymphocyte precursor in the fetal bone marrow or liver. Rag proteins bind DNA at specific recognition sites (recombination signal sequences), where the double stranded DNA is cleaved, and hairpin structures are created. A complex formed of three subunits; DNA dependent protein kinase (Ku70, Ku80 and a catalytic subunit), Artemis, DNA ligase 4 and XRCC4, joins the cut ends together. Terminal deoxynucleotidyl transferase adds extra nucleotides in the junction site, contributing to junctional diversity (Schatz and Ji 2011).

VDJ recombination is an organized process, beginning with rearrangement of the heavy chain, D to J, followed by V-D-J, then followed by rearrangement of the light chain. This process means the clonal repertoire of B cells is generated in a random fashion, resulting in the generation of the maximum amount of diversity possible. The majority of rearrangements generated are non-functional due to errors in reading frames or generation of premature stop codons.

### 1.3.4 Antibody Structure:

![Antibody Structure Diagram](image)

Figure 1-8: Structure of different antibody isotypes, showing monomeric, dimeric and pentameric structures.

There are five different classes of immunoglobulin: IgA, IgD, IgE, IgG and IgM, the structures of which are shown in Figure 1-8. The class of the immunoglobulin is determined by the constant domain of both chains. The class of the immunoglobulin determines its role: memory of primary responses, cell surface or secreted.
IgA is found most commonly in mucosal secretions, in a dimerized form, with an attached J chain. In mucosal secretions it is attached to a secretory piece, protecting it from degradation. Serum IgA is monomeric, with no associated molecules. IgA does not fix complement, but can bind some lymphocytes.

IgG is the most common serum immunoglobulin, and the most versatile: it can fix complement, and bind multiple cell types, via the Fc receptor: macrophages, monocytes, polymorphonuclear leukocytes (PMNs) and some lymphocytes, but not all isotypes of IgG bind the Fc receptor well: IgG2 and IgG4 do not bind to Fc receptors. IgG is secreted as a monomer, with subclasses if IgG differing in hinge length, and number of disulphide bonds. IgG is also the only immunoglobulin capable of crossing the placenta.

IgM has the most complex structure: secreted as a pentamer or a monomer. IgM possesses one more CH4 domain than other immunoglobulins, and covalently binds a J-chain. IgM is the first immunoglobulin to be formed by antigen-stimulated naïve B cells. IgM is capable of fixing complement, and can bind some cells via Fc receptors. IgM can also be a cell surface receptor, lacking the J-chain, but requiring an extra chain of amino acids to tether it to the cell surface.

IgD is a monomeric Immunoglobulin molecule with unclear function, found at low levels in serum.

IgE is a monomeric Immunoglobulin molecule, with an extra CH domain, found in extremely low levels, in serum due to its efficient binding of basophils and mast cells. IgE is involved in control of parasitic infections; however, its ability to bind basophils and mast cells also implicates IgE in allergic reactions.

This study focuses on the IgG molecule. The IgG subclass is produced in T cell mediated immune responses: isotypes are switched, usually from IgM to IgG in the secondary response to antigen. Therefore, IgG forms the dominant subclass of the memory antibodies. Studying the memory subclass of immunoglobulin allows us to determine whether the immune system has been exposed to antigen, in the case of this study, to IA-2.

Each antibody molecule is made up of four polypeptide chains, consisting of two copies of the same light chain (23 kDa each) and two identical heavy chains of approximately 55 kDa each. The polypeptide chains fold into Immunoglobulin domains, and are held together by disulphide bonds between chains (Schroeder and Cavacini 2010). The combination of these chains gives a structure with two separate domains: the Fab domain (fragment antigen binding) and the Fc domain (fragment crystallizable).
A Fab fragment, generated when the Fab domain is cleaved off the Fc (fragment crystallizable), at the hinge region, consists of one light chain, and the VH and CH1 domains of the heavy chain Figure 1-9. Each Fab fragment contains one antigen-binding site. The Fc domain contains the C1q complement activating site that can only activate complement when dimerized with another C1q. The Fc domain is made up of a CH2 and a CH3 domain from a heavy chain.

There are four IgG subclasses found in humans; each of the subclasses possess different numbers of disulfide bonds, and have differing lengths and flexibilities in the hinge region. The structure of the heavy chain determines both the class and the subclass of the immunoglobulin molecule. There are four subclasses of IgG (IgG1, 2, 3 and 4), of which IgG1 is the most common (60% of total IgG) and IgG 4 the least common (4% of total IgG). There are two subclasses of IgA (1 and 2) but no known subclasses of IgE, IgD or IgM.

Each subclass of IgG has a specific role:

IgG1 is primarily involved in the response to protein antigen, and membrane bound protein, capable of triggering effector molecules.

IgG2 is associated with response to bacterial capsular polysaccharide molecules.

IgG3 antibodies are potent effector molecules, capable of inducing a pro inflammatory response

IgG4 antibodies are often produced as a result of long term exposure to allergens.

### 1.3.5 B cell Differentiation:

Only a small proportion of B cells become antigen secreting plasma cells. The most critical factor in determining this outcome is PAX5. PAX5 is expressed throughout B cell development, and in mature
cells, is required to regulate transcription of the immunoglobulin heavy chain, the signal transduction chain Igα and several immune receptors including CD19 and CD21. PAX5 also regulates interferon-regulatory factor 4 (IRF4), IRF8, BACH2 (BTB and CNC homologue 2), Aiolos and SPIB (Nutt, Hodgkin et al. 2015).

Figure 1-10: Schematic of B cell differentiation, from centroblast to memory cell or plasma cell.

As shown in Figure 1-10, the centroblasts in the germinal center upregulate PAX5, which in turn causes increase in expression of Bcl6, and inhibition of BLIMP1. The cell becomes a centrocyte, upregulating NFκB, and IRF4, downregulating Bcl6. The cell at this point can continue to express PAX5 and become a memory B cell, or decrease PAX5, passing further along the path to become a plasma cell. Upregulation of XBP1, BLIMP1, and IRF4 push the cell through the plasma blast phase, to eventually become a mature antibody secreting plasma cell.

Factors required for B cell phenotype include:

1. BACH2, a repressor molecule expressed under regulation by PAX5, throughout development, but switched off in plasma cells.

2. Bcl-6 is expressed in germinal center B cells and plasma cells. Bcl-6 targets BLIMP-1 downstream.

3. OBF1 and OCT2, octamer-binding factors OCT2 and OBF1 which can bind the immunoglobulin gene promoter, but are not required for normal expression of immunoglobulin genes. These factors are required for formation of germinal centers.

4. PU.1, SPIB, ETS1 and IRF8. PU.1 and SPIB form DNA binding complexes in conjunction with IRF4 and IRF8. Both PU.1 and IRF8 are present throughout B cell differentiation; however their function is as yet unknown.
The final differentiation of plasma cells is driven by IRF4, XBP1 and BLIMP1. IRF4 is essential for both germinal center differentiation and terminal differentiation into plasma cells. BLIMP1 expression increases as cells mature: the highest levels are seen in non-cycling plasma cells. A role for XBP-1 has yet to be found, however it is expressed in response to ER stress, which plasma cells are extremely sensitive to.

Following differentiation, the vast majority of plasma cells home to the bone marrow. Sphingosine-1-phosphate receptor 1 (S1PR1) is required for the plasma cells to move into circulation. The chemokine CXCL12, and its receptor CXCR4 can both attract plasma cells to the bone marrow and keep them there (Kabashima, Haynes et al. 2006), along with factors including VLA4, CD44, CD28, CD93, Kruppel Like factor 2, ZBTB20 and Aiolos (Nutt, Hodgkin et al. 2015). Inflammatory cytokines, including CXCL9, CXCL10 and CXCL11 attract plasma cells to sites of inflammation (Odendahl, Mei et al. 2005).

Regulatory B cells are B cells involved in immune regulation. They characteristically express interleukin-10 (IL-10). Loss of regulatory B cells is associated with the development of several autoimmune diseases, including autoimmune encephalitis (EAE), collagen-induced arthritis (CIA), colitis and lupus. Human regulatory B cells express CD48, CD148, CD24, CD27, CD1d, CD19, CD20, CD21, CD23, CD25, and CD38 (Iwata, Matsushita et al. 2011); these cells are thought to develop from a particular set of follicular B cell precursors known as transitional 2 marginal zone precursor (T2-MZP) B cells (Mauri and Bosma 2012).

1.3.6 Avoiding Self-reactive Antibodies:

As the process of development of antibody specificity is random some antibodies will inevitably develop that are reactive to self-antigens. Therefore, during B cell development and maturation any B cell expressing immunoglobulin with a high avidity for a self-antigen is eliminated by apoptosis (clonal selection). This selection ensures that few self-recognizing B cells should escape into the periphery. B cells then migrate to the spleen to develop further, and some previously unseen self-recognizing cells will be destroyed here. Non-self B cells will then progress to either the marginal zone of the spleen or lymphoid follicles of both the spleen and lymph nodes. Follicular B cells can interact with T cells, and form germinal centers: these are clusters of T and B cells which mature and improve B cell immunoglobulin affinity for target antigens by stimulating somatic mutation of V-region genes. Once B cells have a fully developed affinity for antigen, they become activated, and can then differentiate into two different types of B cell: plasma B cells, which circulate in serum and are able to secrete antibody; and memory B cells which remain in the system in case of reinfection.
Antibodies bind specific proteins; the binding of proteins by an antibody then directs processing of epitopes to be presented to T cells (Davidson and Watts 1989). Cell surface antibodies are used to facilitate capture, processing and presentation of specific antigens to T cells, and may play a role in epitope spreading, presenting new determinants to CD4+ T cells. As cells are destroyed, more epitopes may be available for presentation by B cells. B cells therefore influence epitope reactivity in disease. T cells are known to interact with B cells, promoting the expression of less common specific epitopes and thereby break immune self-tolerance of the T and B cell network (Jaume, Parry et al. 2002) leading to development of autoimmune disease.

1.3.7 Antigen Processing and Presentation:
Exogenous antigens captured by antibody are passed into the endocytic MHC class II processing and presentation pathway, whilst endogenous antigen should be passed through proteasomes and presented by the MHC class I molecule.

1.3.7.1 MHC class I pathway:
MHC class I is expressed on all cell surfaces, bar red blood cells. MHC class I presents peptide fragments from intracellular proteins. Peptides are usually self-derived, but in a virally infected cell, MHC class I can present virus derived peptides, and activate cytotoxic T cells. MHC class I molecules are heterodimers of a heavy chain and β2 microglobulin. The heavy chain forms three alpha domains (α1, α2 and α3), and the fourth domain is formed by β2 microglobulin. The peptide-binding site is formed by a groove on the upper surface of the molecule, between the α1 and α2 domains, which can bind peptides of 8–10 amino acids (Hewitt 2003).

1.3.7.2 MHC class II Pathway:
The MHC class II pathway presents exogenous antigen to T cells. It is this pathway that is considered to be most important for islet antigen presentation in Type 1 diabetes because the MHC class II region contains genes conferring highest susceptibility to disease. α and β chains of MHC class II are synthesized in the Golgi, and then translocate to the lumen of the endoplasmic reticulum. In the ER they bind a chaperone molecule, the MHC class II-associated invariant chain. This complex then moves to the endosome, via AP-1. The MHC class II-associated invariant chain is sensitive to proteolysis, and is rapidly degraded in the endosome, leaving a small peptide fragment know as CLIP (class II invariant chain peptide) bound in the MHCII peptide binding groove (Painter and Stern 2012). CLIP is then removed from the peptide binding groove by HLA-DM. Once an antigen has been internalized, it passes into the processing compartments of the B cell, and is digested by Cathepsin enzymes. B cells are known to express Cathepsin B and Cathepsin S (Bryant, Lennon-Dumenil et al. 2002). Peptides from internalized antigen-antibody complexes are loaded onto the antigen binding
pocket of Class II MHC molecules in the late endosome, aided by HLA-DM and HLA-DO. The MHC class II–peptide complex then translocates to the cell surface, for presentation to the T cell receptors of CD4+ T cells.

The MHC class II molecule is capable of binding amino acids contained within antigenic peptides in several locations: the antigen binding groove consists of several pockets, in which specific amino acids can create hydrogen bonds between residues on MHC and peptide. The exact location of the pockets is determined by the MHC allele involved in binding. Four pockets are commonly involved in binding: P1, P4, P6 and P9. Three other pockets are occasionally used: P3, P7 and P10 (Painter and Stern 2012). MHC class II molecules are highly polymorphic, causing binding pockets to vary in their affinities for particular peptides depending on the HLA alleles involved.

1.3.8 Organs of the immune system

1.3.8.1 Central lymphoid Organs
The central lymphoid organs consist of bone marrow and thymus. In these organs, lymphocyte differentiation from stem cells occurs. Lymphocytes, neutrophils, eosinophils, basophils and macrophages are produced in the bone marrow; B cells are differentiated in the fetal liver and later in the bone marrow, whilst T cells are differentiated in the thymus. Lymphocytes migrate from the central lymphoid organs to the periphery.

Both the fetal liver and mature bone marrow contain groups of hematopoietic stem cells which can differentiate into lymphocytes. In the adult bone marrow, both T cells and plasma cells interact with immature B cells allowing maturation of the immune response.

The thymus is a bilobed organ: each lobe consists of a central medulla, containing immature proliferating thymocytes, surrounded by an outer cortex, containing mature T lymphocytes, expressing CD44, permitting binding of the extracellular matrix. Surrounding the cortex are epithelial nurse cells, with interdigitating dendritic cells and macrophages, which present peptide antigens to immature T cells, for thymic selection.

1.3.8.2 Peripheral lymphoid organs
These organs are locations where lymphocytes are able to interact with antigen in the presence of other immune cells. These organs include the lymph nodes, the spleen, and mucosal associated lymphoid tissues, including the tonsils and Peyer’s patches.

The spleen, consisting of both white pulp and red pulp, is both a storage organ for platelets and immune cells, and a secondary lymphoid organ. The red pulp is a zone of macrophages,
lymphocytes, granulocytes and plasma cells. The zone stores platelets and erythrocytes and recycling old erythrocytes. The white pulp is mainly arranged into periarteriolar sheaths, containing T cells surrounding the central arteriole, and B cells in clusters, either as virgin (unstimulated) follicles or stimulated germinal centers, which also contain follicular dendritic cells and macrophages, presenting antigens to B cells.

### 1.3.8.3 The mucosal lymphoid system

There are four major types of mucosal lymphoid organs:

The fat-associated lymphoid tissues, are not encapsulated, nor are they located under a mucosal epithelium. The fat-associated lymphoid tissues may encounter antigen via lymphatic drainage from intestines, and are also thought to directly encounter antigens in the peritoneal cavity via fenestrations in the mesothelial layer (Randall and Mebius 2014).

Tertiary lymphoid tissues are ectopically induced clumps of lymphoid tissue, usually found in the gut or respiratory system, that are formed in response to infection (Carragher, Rangel-Moreno et al. 2008).

Mucosal lymphoid tissues, the gut, genitourinary and respiratory system mucosal surfaces and lamina propria, contain clusters of non encapsulated lymphoid tissue. These clusters can either be solitary, or larger groupings, containing germinal centers, which in the case of the tonsils can also be surrounded by T cell zones. In addition to lymphoid clusters, a large number of dendritic cells, macrophages, lymphocytes and plasma cells can be found. The clusters lack lymphatic vessels, and collect antigen directly from the mucosal surfaces, across the follicle associated epithelium (Mabbott, Donaldson et al. 2013).

Encapsulated draining lymph nodes, are bean-shaped organs, tasked with collecting antigen and antigen-presenting cells from afferent lymphatic vessels that enter the lymph node via the cortical subcapsular sinus, which is lined with macrophages and dendritic cells, capable of filtering lymph, allowing access to the T cell zone (shown in Figure 1-11). The B cell containing follicles are located outside the cortex, whilst the T cell zone is found in the center of the cortex. High endothelial venules bring lymphocytes to the lymph nodes, entering via both T and B cell zones. The lymphocytes travel out of the lymph node via the medullary cords, to the efferent lymphatic vessels.
Figure 1-11: Structure of the lymph node: pathogens, antigen-containing dendritic cells and macrophages reach the lymph node via the afferent lymphatic vessels.

An immune response to a pathogen starts with the encounter of the innate immune system where macrophages and neutrophils are the first cells of the immune system to encounter infectious microorganisms and can control or prevent many bacterial infections. Not all pathogens are recognized by these cells, and, after an initial period where the innate immune system plays an active role in controlling infection, the adaptive immune system provides a more targeted defense, which can also prevent future reinfection. The adaptive immune response begins as the naive B cell first encounters antigen upon entering the periphery. This stage of the response takes place in the germinal centers, located in the cortex of the node, in clusters known as the primary follicles of the peripheral lymphoid organs. The quiescent immature (IgM+IgD+CD38-) B cells transit via the blood. When the cell encounters target antigen (Lanzavecchia 1985; Lanzavecchia and Bove 1985) in the presence of a T cell, they become activated and proliferate. In order to activate, B cells require cross linking of the Fc receptor of the immunoglobulin molecule to occur. Fc receptor cross linking activates a downstream signaling cascade, permitting interaction with T cells (Fearon and Locksley 1996) via peptide presentation on MHC class II.

There are two different stages of B cell activation. The first stage happens in small groups of cell found on the outer edge of the T cell zone. The second stage of activation occurs only to those cells which re-enter germinal centers: this stage of activation leads to the generation of affinity-maturated long-lived plasma cells and memory B cells. Within the germinal center, shown in Figure 1-11, B cells undergo further rounds of somatic mutation. The standard model of germinal center action,
proposed by MacLennan and Gray (MacLennan and Gray 1986), states that upon entering the germinal center, B cells will undergo several rounds of antigen-dependent proliferation. B cells proliferating in the germinal center are called centroblasts. Proliferating centroblasts also undergo high levels of somatic mutation of the Immunoglobulin V-region genes, to increase affinity for antigen. The affinity of the B cell immunoglobulins for antigen is tested by antigen presented on follicular dendritic cells (FDCs). The germinal center generates both high-affinity, long-lived plasma cells, capable of maintaining a high level secretion of antibody and memory B cells, which are B cells circulated in the periphery, many of which then home to the bone marrow, programmed to rapidly differentiate into antigen secreting cells upon re-exposure to antigen. Cells which differentiate into plasmablasts will ultimately become a plasma cell. Plasma cells which do not reenter the germinal center are short-lived, and plasma cells that do return to the germinal center become long lived plasma cells (McHeyzer-Williams 1997). The long lived plasma cells are those which secrete high affinity antibody.

Figure 1-12: Germinal center of a lymph node. CD20 (brown) labelled B cells in the center.

Although class switching is usually initiated in the first, extrafollicular, stage of activation, B cells in the germinal center (follicular) may also undergo class switching of the heavy chain, from IgG and IgM (isotypes present of B cells leaving bone marrow) to generate a suitable isotype (IgA, IgD, IgG) without influencing antigen specificity (Toellner, Gulbranson-Judge et al. 1996; Toellner, Scheel-Toellner et al. 1996). Otherwise, antigen experienced B cells return to a follicle, creating a germinal center, generating long lived memory cells secreting antibody with a high affinity for target antigen.
The follicle then becomes a secondary follicle, with both a germinal center and a mantle zone, with an extra follicular zone, in between which is found the marginal zone.

The peripheral lymph nodes are a secondary form of lymph tissue, monitoring lymphatic fluid drained from peripheral tissues. By monitoring lymphatic fluid, the nodes form an important part of the adaptive immune response: By filtering lymph, macrophages can capture pathogens, and secrete interferons, which can limit pathogen spread. This system of filtration also allows antigen presenting cells to encounter novel antigens and process them for presentation to lymphocytes. Pathogens travel to the lymph node via both circulatory and lymphatic systems. The lymph drains via the subcapsular sinus (SCS) (shown in Figure 1-11) and pools in the medullary sinus as it leaves the lymph node. These sinuses are filled with resident macrophages that can filter pathogens out of the lymph or blood. The sinuses may also hold migrating DCs, which arrive in the lymph node via the SCS and afferent lymphatics, prior to moving into the T-zone to encounter T cells. Other macrophages are found within the medulla and SCS regions (Junt, Moseman et al. 2007).

Whilst the nodes police draining lymph, the node itself provides an environment dedicated to the maturation of T and B lymphocytes. Allowing the production of targeted antibodies, cytokine-secreting effector T helper cells and the activation of cytotoxic T lymphocytes (Ochsenbein, Fehr et al. 1999).

The peripheral lymph nodes are small organs, surrounded by a fibrous capsule, which forms partitions known as the trabeculae, dividing the node into open compartments. The lymph node is constructed as an outer cortex surrounding an inner medulla, leaving an opening at the hilum where blood vessels enter and exit the node. The hematopoietic cells form a complex microarchitecture, where B and T cells are clearly separated in the cortex. B cells are found in primary follicles within the outer cortex (B zone), and can develop germinal centers (GCs) during T cell-dependent immune responses. Different cell types inhabit different zones of the germinal center:

The dark zone, where the original activated B cells become proliferating centroblasts, undergoing somatic hypermutation to generate clones with even greater affinity for antigen.

The light zone, which is filled with antigen presenting dendritic cells and some T cells, where antigenic selection takes place: cells migrating out of the dark zone, having completed somatic hypermutation, are tested for their ability to recognize antigen presented by dendritic cells and to degrade this antigen and present it in peptide form to T cells. In these new B cells, there is a down-regulation of the anti-apoptotic protein Bcl-2, and an increase in expression of pro-apoptotic products such as Fas and Bax. In order to survive, a B cell must be able to bind and present antigen...
to a CD4+ T cell in order to gain the survival signal from the interaction of CD40 and CD154. Interaction with the T cell, via CD40 and CD154 permits the B cell to progress to the next stage of maturation (Bishop and Hostager 2001).

The paracortex houses the T cells, which encounter migrating dendritic cells within this zone. The T zones in peripheral lymph nodes are channeled to allow the distribution of cytokines and other small molecules.

Cooperation between B and T cells is mediated by these members of the TNF family: interaction between the B cell ligand, CD154 and the T cell receptor, CD40, causes activation of multiple transcription activators: including nuclear factor κB (NFκB), nuclear factor of activated T cells (NFAT), B cell specific activator protein (BSAP), AP-1 activating protein 1(AP-1), and the STAT family of signaling molecules. This in turn leads to activation of SAPK, MAPK and NIK and the Lyn, Syk and Fyn protein kinases (Bishop and Hostager 2001; Bishop and Hostager 2003). Should the cell fail to adequately present antigen to the T cell, it will be deleted. Otherwise, the cell can either undergo class switching to become an effector cell, or return to the germinal center for another round of diversification (Kepler and Perelson 1993; Liu, de Bouteiller et al. 1997).

1.4 Development of Type 1 diabetes

Unfortunately, the trigger for autoimmunity in type 1 diabetes remains unidentified. Worldwide incidence varies, and whilst a number of genetic factors are implicated, environmental factors clearly also play an important role (Bluestone, Herold et al. 2010). There are two major risk factors for development of type 1 diabetes: genetics and environment. Several major genetic risk factors have been described, including HLA type, PTPN22 variants, INS-VNTR (IDDM2 locus), UBASH3A and IL1RA (Nokoff and Rewers 2013).

1.4.1.1 Genetics of Type 1 diabetes: The HLA genes

Human Leukocyte Antigen (HLA) class II genes express MHC class II molecules, which are used to present antigens found outside of the presenting cell to T cells. Antigen presenting cells (APCs): B cells, Dendritic cells, mononuclear cells can all act as antigen presenters, and all express MHC class II on the cell surface. GI tract cells (epithelial cells) are also known to express MHC class II and act as APCs. HLA class II molecules are formed of two chains, alpha and beta, which form a heterodimer: each chain is encoded by a separate genetic locus. Three loci encode the most major products of the MHC locus. These are HLA-DR, DQ, and DP.
1. HLA-DP locus: 1 α-chain HLA-DPA1, 1 β-chain HLA-DPB1
2. HLA-DQ locus: 1 α-chain HLA-DQA1, 1 β-chain HLA-DQB1
3. HLA-DR locus: 1 α-chain HLA-DRA, 9 β-chains including pseudogenes. HLA-DRB1-9 (DRB1,3,4 and 5 are functional genes) (Roitt 1998).

DP and DQ molecules each have the added ability to form heterodimers either in cis or in trans: this means that there is added diversity of binding of these molecules.

Prior to antigen presentation, MHC class II molecules form a heterodimer of an alpha and a beta chain, which, in the ER, consists of three MHC class II molecules that must associate with a trimeric complex of CD74. This MHC/CD74 complex enters the endosomal or lysosomal system, and CD74 is degraded by CTSS and CTSL, which leave only a small fragment of CD74, CLIP (Ghosh, Amaya et al. 1995). CLIP is removed from the complex via HLA-DM binding, which then stabilizes the HLA trimer until antigenic peptides are loaded onto the complex.

These peptides can be 10-30 amino acids long, and are loaded into the cleft formed by the complex. Peptides are generated by protease-mediated processing of antigens bound by the APC and passed through the endosomal/lysosomal compartments of the cell. Once loaded, peptides are then presented to CD4+ T cells at the cell surface (Ten Broeke, Wubbolts et al. 2013). Cells of the gastrointestinal tract, such as epithelial cells, express MHC class II molecules and CD74 and act as APCs (Bland 1988). The MHC II molecule bound to a peptide is then transported to the cell membrane surface. In B cells, the formation of the HLA-DM/ MHC class II complex is controlled by HLA-DO (Cresswell 1994). Primary dendritic cells (DCs) also express HLA-DO.

Both development and progression of T1D are closely associated with specific HLA-alleles (HLA-DR4, DR3, and DQ8). The risk conferred to individuals of developing T1D from HLA derives from specific combinations of DR-DQ alleles: there are both high risk and protective DR-DQ combinations. Combinations generating the highest risk of developing disease are DRB1 03:01-DQA105:01-DQB102:01 and DRB1 04:xx-DQA103:01-DQB103:02 heterozygotes. HLA DQB1*0302 is associated with the presence of IA-2 autoantibodies in recently diagnosed T1D children (Sabbah, Savola et al. 1999).

The risk of developing Type 1 diabetes is greater in individuals who have one or more family members affected by the disease. Nearly forty percent of this familial clustering of diabetes is due to inheritance of particular HLA alleles (Concannon, Erlich et al. 2005). The presence of HLA-DR3-DQ2/DR4-DQ8 genotypes confers an increased risk for T1D particularly in individuals who have two
or more autoantibodies (Yamamoto, Deschamps et al. 1998). There is a higher incidence of T1D in DR3/DR3, DR4/DR4 and particularly in DR3/DR4 heterozygous genotypes. This increase is also seen in DR1/DR4, DR1/DR3, and DR4/DR8 genotypes (Noble et al., 1996). Both HLA DR5 and DR2 haplotypes are considered protective, whilst HLA DR7 confers a neutral risk of disease development. HLA-DR competes with DQ to bind peptides: a strong binding low risk DR molecule will therefore outcompete a high risk, weak binding DQ molecule, and prevent presentation of self-antigen (Ge, James et al. 2011).

1.4.1.2 Non HLA class II genes

A study by Noble et al (Noble, Valdes et al. 2010) which genotyped 1753 Caucasian multiplex type 1 diabetes families in order to determine the effect of class 1 MHC alleles, taking into account linkage disequilibrium; the results of this study suggest that at the very least, the B*3906 and B*5701 alleles should be considered as risk factors for disease. There are 73 other loci, thought to influence disease development. Only those for the insulin VNTR, PTPN22, CTLA4, and IL2RA are associated with significant odds ratios. The majority of these other loci are associated with the immune response, supporting the theory that increased risk for Type 1 diabetes is conferred by an aberrant immune response (Concannon, Rich et al. 2009). Polymorphisms in the PTPN22, PTPN2 and UBASH3A (Concannon, Rich et al. 2009) genes cause gain of function of tyrosine-phosphatase signaling, associated with an aberrant immune response, in Type 1 diabetes, and other autoimmune disorders.

A few loci are unconnected with the immune system, but it is believed that some of these may play a role in the response to environmental factors (Cooper, Smyth et al. 2011; Winkler, Lauber et al. 2011). Mutations in SIRT1 the histone deacetylase, has been found to be associated with a family predisposition to Type 1 diabetes (Tang, Zhu et al. 2011).

1.4.1.3 Environmental Risk:

There is a strong genetic association with type 1 diabetes, but genes are not the only factors influencing susceptibility to disease. In studies following identical twins, up to 40% of genetically identical siblings both develop diabetes (Shanmugam, Copie-Bergman et al. 1994), with some studies reporting concordance rates as low as 13% (Kaprio, Tuomilehto et al. 1992). The lack of 100% concordance in genetically identical twins implies a role for the environment, but the specific environmental factors that play a role in disease susceptibility have proved difficult to define. Many potential environmental factors have been implicated in the occurrence of type 1 diabetes. These include several dietary factors (such as gluten and cow's milk), gut flora, sunlight and vitamin D, and viral infection, particularly by enteroviruses.
1.4.1.3.1 **Dietary factors:**
Children who are exclusively breastfed for the first three months of life are less likely to develop antibodies to bovine insulin than those fed a cow’s milk based formula (Vaarala, Knip et al. 1999). It has been suggested that early exposure to bovine insulin increases risk of developing autoimmune response to self-insulin in susceptible individuals (Lefkowith, Schreiner et al. 1990; Oldstone 1990; Oldstone, Ahmed et al. 1990). Analysis of the DAISY (Diabetes and Autoimmunity Study in the Young) cohort suggests that early exposure to cereals may also increase the risk of islet autoimmunity.

As with type 2 diabetes, obesity is linked with increased incidence of type 1 diabetes. The ‘accelerator hypothesis’ suggests that increased weight is associated with an overall increase in diabetes, regardless of type as a consequence of lowered insulin sensitivity. Although several studies have found links between increased BMI and type 1 diabetes, the BABY DIAB study, a study following infants designated with parents diagnosed with type 1 diabetes, for both islet autoantibody and diabetes development, showed no link (Nokoff and Rewers 2013).

1.4.1.3.2 **Vitamin D:**
Sunlight and vitamin D are mentioned as potential environmental factors influencing development of Type 1 diabetes, partly due to the increased incidence of diabetes in Northern Europe (36.5/100,000 in Finland, versus 0.1/100,000 in Venezuela) (Karvonen, Viik-Kajander et al. 2000). However, dietary supplementation of vitamin D is recommended in all northern nations (Knip, Veijola et al. 2005), suggesting that any effect of diminished vitamin D should be compensated for, assuming compliance. However, there is still evidence of vitamin D deficiency in these areas.

1.4.1.3.3 **Viruses**
Viral infection of beta-cells in type 1 diabetes was first reported in 1979, when a strain of Coxsackie virus capable of inducing diabetes in mice was isolated from the pancreas of a type 1 diabetic patient (Notkins, Yoon et al. 1979; Onodera, Ray et al. 1982; Toniolo, Onodera et al. 1982). The presence of virus infected cells within the islet has since been shown by several groups (Morgan and Richardson 2014) and enterovirus RNA can be detected in up to 30% of diabetic patients, but less than 5% of the control population (Knip, Veijola et al. 2005). Enteroviruses include Coxsackie B and rubella; antibodies to Coxsackie B have been found in high levels in type 1 diabetics (King, Bidwell et al.
The immune response to Coxsackie virus B can lead to the destruction of β cells, which could result in uptake of self-antigens by APCs (Horwitz, Ilic et al. 2004). A case of simultaneous development of type 1 diabetes in monozygotic quadruplets, after enteroviral infection, was reported in 2012, with two of the quadruplets developing a viral infection, followed by diagnosis of diabetes simultaneously, and a third showing abnormal glucose tolerance. All four quadruplets tested positive for IA-2 autoantibodies, and GAD65 autoantibodies (Stechova, Halbhuber et al. 2012), underlining the case for viral infection as the trigger for overt type 1 diabetes.

Lonrot et al reported an association between appearance of GAD65 antibodies and enteroviral mRNA, and an increase in enteroviral infection of susceptible children in the six months prior to disease onset (Lonrot, Korpela et al. 2000).

It appears that Type 1 diabetes is not controlled by any one set of genetic or environmental factors: neither have all of the genetic influences nor environmental risk factors been uncovered. A high risk HLA type remains the number one risk factor for disease. Other genes confer only small risk for disease, and environmental risk has yet to be clarified. The risk of development of type 1 must be assessed by taking into account both genetic and environmental factors, including possible gene-environment interactions.

1.4.2 The peripheral immune system in diabetes:

A degree of the autoimmune response against β-cells occurs in the pancreatic lymph node: autoreactive T cells, which have escaped negative selection in the thymus, encounter β-cell antigens presented by dendritic cells. Some of the most important autoantigens recognized by T cells in pre-diabetes are proinsulin and insulin, as demonstrated by Mannering et al (Mannering, Pang et al. 2009). Autoreactive T cells infiltrate the islets via the circulation and establish insulitis initially in the periphery of the islet. It has been shown that T cells within insulitic lesions are specific for islet antigens rather than bystander antigens (Coppieters, Dotta et al. 2012). Pathogenic CD4⁺ T cells migrate to the pancreatic lymph node to mature, and CD8⁺ T cells differentiate into effector T cells in islets and then kill beta-cells, primarily via the perforin-granzyme pathway. Whilst autoreactive lymphocytes and APCs play a major role in the destruction of β-cells, immune cell cytokines and co-stimulatory molecules influence the activation status of the cells. Both IL-1 beta and TNF-alpha are directly toxic to islet β-cells, whilst IL1Ra, MIP-1β, MCP-1 and IP-10 are involved in activation and regulation of inflammation. Pro inflammatory cytokines are believed to contribute directly to killing of β-cells, in part by inducing endoplasmic reticulum stress via the jnk pathway (Brozzi, Nardelli et al. 2018).
Maturation of the immune response within the islet and the PLN is a major contributor to the development of diabetes.

Moriguchi et al (Moriguchi, Sannomiya et al. 2005) showed that by inducing diabetes in rats, an increased flow of lymph could be observed, as well as increased movement of radiolabelled dextran, compared to normoglycemic rats, suggesting that hypoglycemia has an effect of lymphatic transport. The mobilization of lymphocytes was also increased, and the permeability of the lymph nodes also increased; suggesting some damage to the lymphatic system in diabetic rats. Zhang et al showed an overall decrease in CD4+ T cells, and an increase in B cells in pancreatic lymph nodes of diabetic NOD mice (Zhang, Constantinou et al. 1994).

1.4.3 B cells and autoantibodies in diabetes:

B cells play more than one role in the development of type 1 diabetes. B cells produce islet autoantibodies and act as antigen presenting cells, stimulating activation of autoreactive T cells (Figure 1-13). Transgenic NOD mice only capable of producing cell surface IgG, without the ability to secrete antibody, still develop diabetes, although at a lower rate than wild type NOD mice (Wong, Wen et al. 2004). Maternally transferred (cord blood) autoantibodies may also play a role in development of disease, in both the NOD mouse (Greeley, Katsumata et al. 2002) and humans (Lundgren, Lynch et al. 2015), suggesting the role of cell surface and secreted antibody in disease development is potentially important but not altogether clear.
Figure 1-13: Scheme showing the role of the B cell in the immune response: B cells recognize and take up soluble antigen from β-cells, which is the internalised and digested. The B cells acting as APCs present antigen derived peptides to T cells on HLA class II molecules, thus stimulating T cell activation, diversification of the immune response and destruction of target cells expressing antigenic peptides.

The role of B cells in both exacerbating and controlling autoimmune disease is complex. As mentioned previously, antibodies to CD20 (Rituximab) can prevent or slow progression of diabetes in both mice and man (Hu, Rodriguez-Pinto et al. 2007; Hu, Ding et al. 2013). The expression pattern of this molecule can provide some clues to the activity of the B cell in autoimmune disease: CD20 expression is down regulated once B cells differentiate into plasma cells, suggesting that the actions of immature effector B cells is a critical factor in autoimmune disease. Use of Rituximab in a variety of autoimmune diseases has shown not only that improvement in symptoms can occur before a reduction in serum antibody levels (Martin and Chan 2006), but that in some cases, symptoms of disease are exacerbated by removal of B cells, where patients develop psoriasis after rituximab treatment for rheumatic diseases (Dass, Vital et al. 2007). Neutralization of both plasma cells and prevention of B cell proliferation does not improve outcomes in rheumatoid arthritis, another autoimmune disease (Genovese, Kinnman et al. 2011). These studies suggest that B cells may play several roles in autoimmune disease, some of which may be antibody independent. Alongside autoantibody production, B cells present antigen, release cytokines and are believed to play a role in regulation of the immune system.
Secondary actions of B cells in disease include secretion of pro-inflammatory cytokines, including IFN-γ. Interferon-γ may inhibit development of Treg (regulatory) T cells, thereby enhancing inflammation, and speeding disease progression. Olalekan et al (Olalekan, Cao et al. 2015) show that in a mouse model of arthritis, depletion of B cells leads to an increase in differentiation of naïve CD4+ T cells into Tregs, where interferon-γ secretion would usually modulate inflammation and promote TH1 cell differentiation. B cell cytokine production defines two effector subsets of B cells, known as Be1 and Be2. These cells secrete distinct patterns of cytokines, determined by cytokines present in the environment, when cells first encounter antigen and T cells. Be1 cells secrete IFN-γ and IL-2 in the presence of Th1 cells, whilst Be2 cells secrete IL-4 and IL-6 in the presence of Th2 cells.

Non IgG B cells may also play a role in disease development: recent studies of anergic (Bnd) IgM and IgD cells suggest a transient loss of these cells in type 1 diabetes, suggesting a possible failure of regulation (Smith, Packard et al. 2014).

B cell autoantibody targets in humans identified to date include: (Pro) insulin, IA-2, GAD65 and Znt8. Other autoantibodies are known to exist (Perego 2012; Wenzlau and Hutton 2013), including autoantibodies directed to Tetraspanin 7, carbonic anhydrase, lactoferrin, pancreatic islet monosialo-ganglioside (GM2-1), imogen 38, peripherin and HSP (heat shock protein). Although some of these autoantibodies have been identified, most remain to be fully characterized (Winter and Schatz 2011; Pietropaolo, Towns et al. 2012). These autoantigens also probably represent targets of T cell autoimmunity in diabetes, and there may be significant levels of T-B cell cooperation in disease pathogenesis. There are similarities in autoantibodies identified in NOD mice that can also be found in humans (Giarratana, Penna et al. 2007). However, IA-2 is not one of the major autoantigens in the NOD mouse.

The four major autoantigens in diabetes are components of the insulin secretory system, and are all located in secretory vesicles: IA-2, Insulin and ZnT8 are all located in the membrane of the insulin secretory vesicle, whereas GAD65 is localized to synaptic like vesicles in the β-cell (Arvan, Pietropaolo et al. 2012):

The major role of B cells in pathogenesis of diabetes is thought to be as professional antigen presenting cells. Studies in the NOD mouse have demonstrated that NOD CD4+ T cells are in the majority stimulated by B cells, as defects are present in the macrophage and monocyte antigen presentation systems, limiting their ability to activate T cells (Serreze, Gaskins et al. 1993).
There are several well characterized β-cell autoantigens in T1D:

1. Insulin, antibodies to which can only be measured accurately prior to disease onset, as they are masked by antibodies to insulin developed shortly after starting insulin therapy.
2. IA-2 (insulinoma associated protein) is a member of the protein tyrosine phosphatase family, associated with early onset and pre-diabetes.
3. Glutamic acid decarboxylase 65 (GAD65) antibodies persist for some time after disease onset.
4. Zinc transporter 8 (ZnT8) (Wenzlau, Juhl et al. 2007) are also associated with early onset of diabetes; these antibodies usually disappear quite rapidly after disease onset.

Although insulin autoantibodies may seem the most important markers of disease onset, measurement of these antibodies has been less reliable, although with the development of commercial ELISA assays, this should improve. Samples for measurement must be taken within one week of beginning exogenous insulin therapy, and assays to measure insulin autoantibodies from later onset patients remain problematic (Achenbach, Guo et al. 2010). Tetraspanin-7 has only very recently been identified. Therefore, GAD65, IA-2 and ZnT8 have become the most commonly studied autoantigens. Improvements in screening have allowed the development of reliable IAA assays, as used in the TEDDY (The Environmental Determinants of diabetes in the Young) study, testing for autoantibodies in at-risk children (deemed at risk by high risk HLA) (Krischer, Lynch et al. 2015).
It is common to screen for IA-2, GAD65 and Znt8 antibodies at the point of disease diagnosis (Tiberti, Yu et al. 2011). Presence of autoantibodies is most pronounced in young, recent onset patients: in a study of newly diagnosed children in Finland, 91% of children tested were reactive to at least two autoantigens, and 71% were reactive to three autoantigens. 0.5% of non-diabetics, and up to 4% of non-diabetic relatives of patients express these antibodies (Knip, Korhonen et al. 2010). Risk of developing diabetes increase with the number of autoantigens recognized, rather than the titre of autoantibodies. The DPT-1 trial showed that the 5 year risk for development of Type 1 diabetes is as follows (Winter and Schatz 2011):

- 1 autoantibody: 25%
- 2 autoantibodies: 70%
- 3 autoantibodies: 80%

Antibodies to IA-2 were found in 86% of children tested (Savola, Bonifacio et al. 1998). The importance of IA-2 as an autoantigen is demonstrated by the presence of IA-2 reactive autoantibodies months and years before overt symptoms appear (Kawasaki, Eisenbarth et al. 1996; Morran, Casu et al. 2010). IA-2 autoantibodies may mark more rapid progression to diabetes than GAD65 autoantibodies, for example, and are often detected along with other autoantibody specificities. The presence of islet autoantibodies, coupled with studies in man and mouse, may help to explain how selective β-cell loss in type 1 diabetes occurs.

### 1.4.4 Screening for Type 1 diabetes

As genetic screening improves, a combination of HLA Typing, screening for common polymorphisms, and autoantibody screening may be used to predict disease. Currently, testing for the presence of autoantibodies remains one of the best markers for disease.

Whilst insulin autoantibodies were discovered in 1963 (Pav, Jezkova et al. 1963), the presence of islet autoantibodies was not widely accepted until the demonstration of circulating antibodies to islet cell antigens by indirect immunofluorescence on pancreatic tissue sections in 1974 (Bottazzo, Florin-Christensen et al. 1974). The discovery that patients produce antibodies to islet autoantigens supported the concept that Type 1 diabetes is an autoimmune disease. Islet autoantibodies are important biomarkers of disease progression - the presence of serum immunoglobulins or antibodies to specific islet cell or insulin molecules is commonly used to predict disease (Orban, Sosenko et al. 2009; Fuchs, Adler et al. 2012) and is associated with disease onset. Another marker, HbA1C is commonly used to determine poor metabolic control, associated with ongoing disease (Eisenbarth,
Gianani et al. 1998). Other less useful disease biomarkers include autoantibodies to endocrine tissue epitopes and cytokine profiles.

Screening is performed using either radiolabeled target antigen (Insulin, GAD65, ZnT8 or IA-2), which is incubated with patient sera, washed, and the quantity of radioactivity remaining bound to patients antibodies is assessed, to determine antibody positivity or using ELISA, a non-radioactive colorimetric assay.

The four autoantibodies commonly screened for are those to insulin (IAA), Glutamic acid decarboxylase (GAD65A), Islet autoantigen 2 (IA-2A) and Zinc transporter 8 (ZnT8A). 80% of patients present with islet autoantibodies, with 60% of children producing Insulin autoantibodies and or IA-2A, 63% with Znt8 and 70-80% of individuals with GAD65A. Numbers of patients presenting with autoantibodies vary across studies and populations: Caucasian patients have been reported as showing rates of 65-84% positivity for GAD65 antibodies, whilst Asian type 1 diabetes patients show greater variations, between 5-83.3% (Zimet, Rowley et al. 1993; Schmidli, Colman et al. 1995; Akamine, Komiya et al. 1997). The IA-2 autoantibody is present in approximately 60% of recent-onset type 1 diabetes patients, with less inter-study variation (Gorus, Goubert et al. 1997; Vandewalle, Falorni et al. 1997). Testing for ZnT8 autoantibodies also identifies 60-80% of patients (Wenzlau, Juhl et al. 2007).

The latest data on screening for biomarkers of Type 1 diabetes suggest that metabolic profiling, in particular screening for lower levels of methionine, can contribute to assessment of diabetes risk in children as metabolic markers associate with islet autoantibodies (Pflueger, Seppanen-Laakso et al. 2011).

1.4.5 Models of Type 1 diabetes

In vivo studies of type 1 diabetes require appropriate animal models to mimic both onset and pathogenesis of the disease. Several systems exist for the generation of animal models of type 1 diabetes:

1. Chemical ablation of the islets of Langerhans (alloxan, streptozotocin, Ferric nitrilotriacetate and dithizone). These techniques are traditionally used to test transplantation therapies, β-cell regeneration and novel antidiabetic drugs or insulin formulations (Federiuk, Casey et al. 2004; Jederstrom, Grasjo et al. 2005; Sheshala, Peh et al. 2009).

2. Virus-induced diabetes, particularly coxsackie viruses, thought to possibly play a role in triggering human type 1 diabetes (Jaidane, Sane et al. 2009).
3. Genetic modification of the animal to enhance susceptibility to the disease, using AKITA mice, which have a mutation in the INS-2 gene, generating large quantities of unfolded protein in the β-cell, inducing ER stress and β-cell death. These mice have an extremely low β-cell mass, making the model a viable alternative to streptozotocin treated animals in transplantation studies (Mathews, Langley et al. 2002).

4. Naturally occurring spontaneous disease developing animals: of these, the most commonly used models are those that spontaneously develop the disease: non-obese diabetic (NOD), Bio Breeding rat (BB), Long-Evans Tokushima lean rat (LETL), Komeda diabetes-prone rat (KDP) and Lewis IDDM (LEW-IDDM) rats.

5. Humanized mouse models: NOD-scid mice are immunodeficient, therefore can be engrafted with human hematopoietic and immune cells. This can be combined with grafted human islets, to better mimic the human condition (Greiner, Brehm et al. 2011).

6. HLA transgenic mice: HLA-A2 transgenic mice recognize antigens targeted by human CD8+ T cells (Jarchum, Baker et al. 2007).

Whilst rodent models are commonly used to model type 1 diabetes in the laboratory setting, other larger animal models exist, including naturally occurring canine diabetes. Although animal models have greatly enhanced our understanding of type 1 diabetes, animal models differ significantly from humans, both in the immune response to islet autoantigens, structure of the islet, degree and composition of infiltration and gene expression (Brissova, Fowler et al. 2005). The generation of more humanized models will greatly enhance research in this area.

1.4.6 Prevention of Type 1 diabetes

There is a long history of intervention trials in Type 1 diabetes, attempting to halt the progression of disease: beginning with the partially encouraging cyclosporine study (Stiller, Laupacis et al. 1983), followed by the unsuccessful DPT-1 (Diabetes Prevention Trial-1) trial in US, attempting to use insulin administration prior to disease onset. Although the DPT-1 trial itself was unsuccessful, it demonstrated a possible improvement in insulin secretion in subjects given oral insulin. This was followed up with an intra-nasal trial, which showing an increase in circulating anti insulin antibodies, and a decrease in insulin reactive T cells (Harrison, Honeyman et al. 2004) in at risk patients. A follow up study, of new onset diabetic patients, demonstrated a decrease in the antibody response to exogenous insulin, and a decrease in insulin reactive T cells, which the authors suggest is indicative of tolerance (Fourlanos, Perry et al. 2011), and is now being repeated, using a higher dose in both Point and PrePoint studies (Primary intervention with oral insulin for prevention of type 1 diabetes in
infants at high genetic risk to develop type 1 diabetes) (Skyler 2013). Following these trials, different targets were chosen.

**1.4.6.1 Immune intervention therapies:**

These trials attempt to block the inflammatory destruction of pancreatic β-cell, using immunosuppression:

1. Etanercept (anti TNF-α), showed improvements in HbA1c, in a small study of 18 patients. Increased levels of TNF-α are seen in Type 1 diabetes, suggesting that there is chronic inflammation: anti-TNF-α would prevent this inflammation, and is an established treatment in other autoimmune diseases, including rheumatoid arthritis. However, Etanercept causes severe side effects, and is therefore not recommended for use in children. A LADA patient has been successfully treated with Etanercept for 11 years, gaining normal HbA1C levels (Skyler 2013).

2. Atorvastatin (a statin) was trialed after studies showed that statins, in combination with insulin therapy, preserved β cell function in mouse models. Atorvastatin is an anti-inflammatory, cholesterol lowering statin. In the DIATOR trial, Atorvastatin treated patients showed slower declines in both fasting and stimulated c-peptide levels (Martin, Herder et al. 2011).

3. Blockade of the innate immune modulator, IL1: IL1 is a pro-inflammatory cytokine believed to influence apoptosis and aggravate β cell death in Type 1 diabetes. To block the action of IL1 receptor antagonists (IL1-RA) were used. Two compounds, Anakinra and Canakinumab were used in two blinded multicenter trials; these compounds demonstrated no adverse effects, but there was no improvement in c-peptide in patients receiving treatment, suggesting that IL1-RA are ineffective as single drugs in the prevention of Type 1 diabetes (Moran, Bundy et al. 2013).

4. Abatacept is a human monoclonal antibody which is capable of blocking co stimulation of T cells. In 2011 77 of 122 patients received Abatacept, results demonstrated a sustained improvement in HbA1c levels in patients who had received Abatacept, rather than placebo, even several years after the termination of the trial (Orban, Bundy et al. 2011).

5. Alefacept, a T cell modulating drug used in treatment of psoriasis, which disrupts CD2-mediated T cell co stimulation and depletes T cells via an NK cell dependent mechanism caused showed an increase in circulating c-peptide and a decrease in circulating T cells when used in treatment of type 1 diabetes (Rigby, Harris et al. 2015).

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1.4.6.2 **Immune cell specific therapy:**

Anti CD3 antibodies target the general population of T cells, as a general immunosuppressive regimen; as the T cell is believed to be the instigator of β-cell death in type 1 diabetes, depletion of these cells in the pre onset or early onset stages of disease could prevent further damage to the islet and preserve some degree of β cell function.

Use of cyclosporine, an inhibitor of T cell growth and effector T cell function can increase levels of remission from T1D (Feutren, Papoz et al. 1986). Cyclosporine binds cyclophilin inhibiting interleukin function and cytokine release, reducing activity of effector T cells. Administration of cyclosporine to recent onset patients allowed partial recovery of β-cell function (De Filippo, Carel et al. 1996). However, the drug was less effective in younger patients, and ultimately toxicity issues, particularly in the kidney, caused it to be withdrawn from trials (Keown and Stiller 1987; Stiller, Dupre et al. 1987).

Teplizumab (Fc receptor-nonbinding anti-CD3 monoclonal antibody) (Herold, Gitelman et al. 2013): At the two year follow up point, following treatment at two weeks and one year, patients show an average 75% improvement in fasting c-peptide levels, suggesting some level of preservation of β-cell function. Unfortunately, not all subjects respond to Teplizumab treatment.

Otelixizumab: Early trials of Otelixizumab showed improved circulating c-peptide levels, indicating a possible slowing of disease progression, but produced severe side effects. The DEFEND-2 used a 3.1mg dose, administered over 8 days. Unfortunately, both this trial and DEFEND-1 had to be terminated early, as no improvement in c-peptide was seen, and side effects were severe (Ambery, Donner et al. 2014).

1.4.6.3 **Antigen specific T cell therapy:**

General immunosuppressive regimens can have deleterious effects on a patient’s immune system, therefore in more recent studies; attempts have been made to develop more easily administered, specific therapies. A study examining the treatment of recent onset patients with Diamyd, (GAD-Alum, containing alum as an adjuvant, in order to increase immune recognition) showed a decrease in the rate of decline in c-peptide (Ludvigsson, Faresjo et al. 2008). Unfortunately, a follow up study showed no difference between treated and untreated groups and a multicenter phase III study in patients failed to reproduce these promising results (Ludvigsson, Krisky et al. 2012).

Results of these studies can be difficult to interpret: a recent study using synthetic peptide, DiaPep277, derived from the 437–460 sequence of the human 60 kDa heat shock protein (HSP60) was retracted in early 2015 (Raz 2015): treatment of NOD mice with DiaPep shows a slowing or
complete stop of the autoimmune destruction of β-cells. Results of phase 2 trials in adults show an increase in stimulated c-peptide in all treated patients, despite the placebo group entering the trial with higher stimulated c-peptide (Raz, Elias et al. 2001).

### 1.4.7 B cell therapies in Type 1 diabetes

Although type 1 diabetes is considered to be T cell mediated, treatments targeted at B cells have also been trialed, using B cell depletion with anti-CD20 therapy (Rituximab). CD20 is a phosphoprotein expressed on the cell surface of more than 95% of B cells. As CD20 acts as a calcium channel, allowing differentiation and proliferation, binding of the CD20 channel prevents cells from expanding. Only plasma cells are unaffected. Anti-CD20 treatment in recent onset patients decreases titres of insulin antibodies although there was no effect on GAD65, IA-2 or Znt8 antibody titres (Yu, Herold et al. 2011). The TrialNet phase 2 clinical trial demonstrated that a single course of rituximab could slow disease progression for over a year in recent onset patients. This course was also shown to reduce the concentration of autoantibodies in patient sera. However, the two year follow up shows no long lasting effect on beta cell function or disease treatment (Pescovitz, Greenbaum et al. 2009; Pescovitz, Greenbaum et al. 2014).

However, a non-specific treatment such as anti-CD20 therapy can have adverse effects on the immune system as a whole. Therefore, identifying specific immunogenic epitopes would permit the generation of a more specifically targeted therapy. These results suggest that B cells as well as T cells may also play a key role in the development of T1D, acting as professional antigen presenting cells, recruiting and activating CD4+ T-lymphocytes. Identification of specific epitopes of autoantigens recognized by B and T cells would aid the development of specific inhibitors to B cells which recognize diabetogenic autoantigens.

Studies performed in transgenic BDC2.5NOD mice (Xiang, Peng et al. 2012) showed an increase in B cells with a regulatory (CD1d (-)) phenotype as B cells regenerated after depletion using anti CD20. The results suggest that the increase in regulatory B cells may be responsible for improvement in symptoms of diabetes after anti CD20 therapy. A follow up study performed using anti CD20 treatment in combination with anti-CD3 can delay onset of type 1 diabetes in pre diabetic BDC2.5NOD mice (Hu, Rodriguez-Pinto et al. 2007; Hu, Ding et al. 2013).

Henry et al (2012) blocked antigen presentation by insulin specific B cells in NOD (Non obese diabetic) mice, using a monoclonal anti-insulin antibody to bind antigen on the BCR. Cross linking of the BCR causes deletion of the B cell in the periphery (Ait-Azzouzene, Verkoczy et al. 2006) to maintain or reinstate tolerance if the number of insulin reactive B cells is low. When the number of
circulating insulin reactive B cells is high, administration of insulin antibody is insufficient to prevent disease onset. Introduction of an IgG null gene into NOD mice also prevents onset of diabetes (Forsgren, Andersson et al. 1991).

1.5 Autoantigens in Type 1 diabetes:

Autoantigens in diabetes are not only found in β-cells, however, in type 1 diabetes, only the β-cells are targeted, suggesting a disconnect between the immune targeting of these antigens in the pancreas, when compared with other tissues.

1.5.1 Insulin

Autoantibodies to insulin were discovered in 1963: following this discovery, the identification of T cell specific destruction of islet β-cells by Huang et al (Huang and Maclaren 1976) brought the focus to the insulin molecule. Pro-insulin has been identified as the first self-antigen recognized in the initiation of diabetes in the NOD mouse (Krishnamurthy, Dudek et al. 2006), and insulin is among the first autoantigens recognized in type 1 diabetes. Furthermore, polymorphisms within the insulin VNTR (variable number of tandem repeats) are risk factors for development of type 1 diabetes (Brezar, Carel et al. 2011).

One immunodominant peptide B9-23, from the B chain of the insulin molecule, is immunogenic in the NOD mouse (Daniel, Gill et al. 1995; Wong and Janeway 1999), and is recognized by autoreactive T cells in human subjects (Alleva, Crowe et al. 2001). This peptide is also found as a naturally processed product of insulin in the β-cell secretory granules, and can be presented by dendritic cells to T cells. T cells recognizing a peptide from the insulin A chain (amino acids 1-15) have been found in the peripheral lymph nodes of type 1 diabetics (Kent, Chen et al. 2005).

Whilst insulin autoantibodies can be detected in new onset type 1 diabetic patients, these antibodies are rarely detected in older patients (Rapoport, Mor et al. 1998). The implication that insulin reactivity plays a key role in disease development is underlined by the ability of isolated insulin reactive T cells to transfer disease between mice (Daniel, Gill et al. 1995). CD4+ T cells isolated from type 1 diabetic patients have been shown to recognize epitopes from insulin A and B chains (Schloot, Willemen et al. 1998). Oral, intranasal or subcutaneous insulin administration has been shown to protect NOD mice against disease onset.

Pre-Proinsulin (PPI) has also been identified as a target for autoimmunity in type 1 diabetes: Baker et al (Baker, Petrich de Marquesini et al. 2008) demonstrated reactivity of Type 1 diabetic CD8+ cells to a range of more than 50 epitopes across the PPI molecule, some of which had extremely low
predicted affinity with patient HLA type, suggesting many low affinity epitopes could also be activating the CD8+ T cell response in patients.

1.5.2 Glutamic acid decarboxylase:
Glutamic acid decarboxylase (GAD) synthesizes gamma amino butyric acid, an inhibitory neurotransmitter. GAD65 was identified at one of the major target of islet autoimmunity in 1990 (Baekkeskov, Aanstoot et al. 1990), and was previously identified as a major target for autoimmunity in stiff person syndrome (Solimena, Folli et al. 1988). There are two forms of GAD (glutamic acid decarboxylase): one of 65kDa, and one of 67kDa. The 65K isoform of GAD is targeted to the Golgi apparatus and peripheral synaptic like vesicles in the cell. In the pancreatic β-cell, the 67K isoform is targeted to the cytoplasm, and can only associate with membranes of vesicles in the presence of the 65k isoform (Kanaani, Cianciaruso et al. 2015).

Autoreactivity to the 65kDa isoform is associated with the development of type 1 diabetes; this is also the isoform recognized in stiff person syndrome, suggesting development of autoimmunity to the 67k isoform is likely to be secondary to a primary response to GAD65. There are three epitopes on GAD65 located in the central and C-terminal domains of the protein, and a linear epitope in the N-terminus. Epitopes were identified using monoclonal anti islet cell antibodies, derived from a recent onset type 1 diabetic patient. The group used a combination of N and C-terminal deletion mutants, in combination with protein footprinting to identify which epitope regions of GAD65 were recognized by patient antibodies. This study identified middle and C-terminal epitopes (Richter, Shi et al. 1993). A follow up study, using novel monoclonal patient derived antibodies, identified an N-terminal epitope (Syren, Lindsay et al. 1996). Central domain epitopes are conformational, one from amino acids 240 to 435 and a second from amino acids 451 to 570 (Powers, Bavik et al. 1999). The C-terminal domain encompasses amino acids 445-585, and the N-terminal domain amino acids 1-99. Studies suggest that reactivity to GAD65 changes with disease onset (Falorni, Ackefors et al. 1996), with reactivity beginning with recognition of middle region epitopes, followed by spreading to C-terminal epitopes, and finally N-terminal epitopes, with less frequent spreading of reactivity to GAD67 (Bonifacio, Lampasona et al. 2000). Identification of a mutant form of GAD65, with four amino acid substitutions, inspired an investigation into the importance of these amino acids in antigen recognition in type 1 diabetes. This study used site directed mutagenesis to reveal that substitution of both Asparagine 247 and Leucine 574 could inhibit binding of monoclonal antibodies to either the c-terminal or n-terminal epitope, and inhibit binding of patient sera. Substitution of Asparagine 247 alone had no effect on monoclonal antibody binding, whilst substitution of Leucine 574 inhibited binding of antibodies to the c-terminal epitope (Tree, Morgenthaler et al. 2000).
The presence of autoantibodies to the GAD65 antigen is associated with the expression of HLA-DR3-DQ2. Epitope recognition in the GAD65 autoantigen has been well characterized in diabetes; studies have demonstrated that the DQA1*03-DQB1*0303 HLA type is more commonly found in individuals recognizing central and middle epitopes of the GAD65 antigen. Analysis of the structure of both isoforms of the GAD protein show that the 65kDa isoform has a more flexible, charged C-terminus, with potentially antigenic PLP binding site surrounding the middle regions, making the 65kDa isoforms more antigenic than the 67kDa isoforms (Arafat, Fenalti et al. 2009). Knowledge of both the structure and location of the epitopes within the autoantigen is essential to the understanding of the development of autoimmunity.

1.5.3 Zinc transporter 8

ZnT8 was identified as a major autoantigen in type 1 diabetes by microarray analysis of human and rodent islet cells (Wenzlau, Juhl et al. 2007). The ZnT8 antigen is transmembrane zinc efflux channel, located in the membrane of the insulin granule of the β-cell. The protein is encoded by the SLC30A8 gene. Autoreactivity of B cells to the antigen was tested using radioligand binding assays, which revealed 63% of new onset diabetics were reactive to the protein in this study. ZnT8 autoantibodies occur more frequently in patients expressing the HLA-DR13-DQB1*0604 haplotype than other haplotype, although it must be noted that this HLA type confers a neutral risk for type 1 diabetes (Salonen, Ryhanen et al. 2013). The antibody recognition of ZnT8 in type 1 diabetes is interesting, as patients recognize three epitope variants of ZnT8 caused by the presence of a single nucleotide polymorphism (snp), rs13266634 at position 325 in the protein sequence. The snp changes native ZnT8 from arginine (R) to tryptophan (W) at position 325. There is a third polymorphism which is rare in all populations, changing arginine to glutamine (Q). There are three variants of ZnT8, specifically arginine (R), tryptophan (W) or glutamine (Q). Patients may recognize epitopes alone or in combination. This snp is associated with development of both type 1 and type 2 diabetes (Sladek, Rocheleau et al. 2007; Achenbach, Lampasona et al. 2009). Type 1 diabetic patients expressing the R encoding C version of the allele are more likely to produce antibodies to the R (arginine) variant, whilst those with the T allele more commonly develop antibodies to the W (tryptophan) variant (Wenzlau, Liu et al. 2008). The C/C variant is more commonly associated with a younger age at onset, and both homozygous variants are thought to be associated with higher risk of disease development.

1.5.4 The IA-2 autoantigen:

IA-2 (also known as PTPRN) and its relative, IA-2β (PTPRN2) are neuroendocrine proteins, predominantly located in the membrane of the secretory granule of the β cell (Solimena, Dirks et al. 1996; Torii 2009). Autoantibodies to these proteins were first detected by studying reactivity to
tryptic fragments of proteins from islet cells and their identity as the tyrosine phosphatase-like proteins was uncovered by Payton and Hawkes (Payton, Hawkes et al. 1995). The group developed radiolabeled transcripts representing both the central region (ICA512E), and the intracellular domain (IA-2IC) of the molecule, and demonstrated that recognition of the 40000Mr islet autoantigen could be inhibited by addition of an excess of IA-2-IC protein, implying that the 40000Mr protein and IA-2 were one and the same. Antibodies able to bind IA-2IC could also immunoprecipitate the whole IA-2 protein, including the extracellular domain. However, addition of excess cytoplasmic domain protein prevents binding of the whole protein, showing that all reactivity is most likely directed to the intracellular domain.

Figure 1-14: Linear diagram of the 979 amino acid protein tyrosine phosphatase IA-2, showing major regions of antibody reactivity: the juxtamembrane domain (JM, amino acids 601-682), central region (827-860) and C-terminal epitopes (878-979).

The IA-2 gene is located on chromosome 2q35, and encodes a 110kDa transmembrane protein. Post translational modification of the protein leads to the removal of the signal peptide by cleavage at two dibasic cleavage sites, and N-linked glycosylation, yielding a mature protein of 64kD (Hermel, Dirkx et al. 1999). IA-2 is formed of 979 amino acids, shown as a linear representation in Figure 1-14. This is composed of a signal peptide (amino acids 1–24) followed by an extracellular domain, 25–576, trans-membrane domain 577–600, and the intracellular 601–979 domain, which is divided into three separate regions, Juxtamembrane (JM), Central region, and C-terminal region.

Neither IA-2 nor IA-2β are thought to have enzymatic activity as there are two key differences in sequence in the PTP domain to the sequence of functional protein tyrosine phosphatases; substitution of amino acids 911 and 877 restores functionality (Magistrelli, Toma et al. 1996). IA-2 and IA-2β are protein tyrosine phosphatase-like proteins containing a region which is homologous to
the catalytic domain of receptor type protein tyrosine phosphatases (RT-PTPs). The PTP domain of the molecule is intracellular, located on the cytosolic side of the secretory granule membrane; both IA-2 and IA-2β are known as dense core vesicle proteins.

Although no role has yet been proven for the IA-2 protein, it is believed to act in both the regulation of granule insulin content, and β-cell growth and proliferation (Torii 2009) as reports from studies using IA-2 knockout mice show fewer dense core vesicles (Cai, Hirai et al. 2011), reduced glucose tolerance and lower levels of insulin secretion (Mziaut, Trajkovski et al. 2006). It has been suggested that the intracellular cleaved fragment of IA-2 can then translocate to the nucleus and upregulate transcription of both insulin and IA-2 genes (Trajkovski, Mziaut et al. 2008). The IA-2 antigen has been localized to the secretory granule of the β-cell and other neuroendocrine cells, including in the brain, pituitary and adrenals (Solimena, Dirkx et al. 1996). The intracellular domain of IA-2 domain binds βIV spectrin (Berghs, Aggujaro et al. 2000), β2-syntrophin and f-actin associated protein, which is believed to play a role in subcellular localization of membrane proteins including sodium channels, ATP-binding cassette transporters and potassium channels by binding via its PDZ domain to the C-terminal PDZ binding site (Constantin 2014). It is thought to play a role in granule docking and loading by connecting the secretory granules to the actin cytoskeleton within the cytoplasm. When insulin secretion is stimulated, the IA-2 molecule is thought to be cleaved, leading to mobilization of the insulin secretory granule and docking with the plasma membrane (Ort, Voronov et al. 2001). The JM domain of IA-2 contains a PEST sequence, from amino acids 644 to 653, allowing calcium dependent calpain cleavage of the protein at this location, removing the majority of the JM domain. There are 2 PDZ sequences, the first of which is located between amino acids 659-696, and the second at the c-terminus of the molecule, from amino acids 950-979 (Ort, Voronov et al. 2001). β2 syntrophin binding of the JM domain PDZ site has been shown to regulate calpain cleavage of the JM domain.

1.5.5 IA-2 splice variants

IA-2 is expressed as at least four alternatively spliced variants. All lymphoid organs express a variant lacking exon 13, which codes for the transmembrane domain and JM domain (Diez, Park et al. 2001) of the molecule. The splice variant expressed in thymus and spleen lacks residues 557-629. Another splice variant, lacking exon 14, is also expressed in some pancreases. Differential expression of splice variants of the IA-2 molecule between organs could lead to a failure to induce immune tolerance to both linear and conformational epitopes within the molecule, if they are not expressed in the lymphoid organs (Diez, Park et al. 2001) but are expressed in the pancreas.
1.5.6 The role of IA-2 as an autoantigen in type 1 diabetes:
Both IA-2 and IA-2β are protein tyrosine phosphatase family members. Although the proteins share 74% homology, IA-2 appears to be the dominant antigen in type 1 diabetes, with immunity to IA-2β occurring later in disease, possibly due to cross-reactivity between the two structurally similar proteins, followed by epitope spreading to generate IA-2β-specific responses.

The presence of maternal autoantibodies to IA-2 in cord blood correlates strongly with later disease development in the DiPiS (diabetes Prediction in Skåne) study, underlining the importance of this autoantigen (Magistrelli, Toma et al. 1996). Cord blood autoantibodies to either GAD65 or insulin have no effect of future disease development. The development of antibodies to the JM domain of IA-2 is significantly associated with the rapid development of type 1 diabetes, in the absence of high risk HLA types (Naserke, Ziegler et al. 1998), even though antibodies to IA-2 JM associated positively with HLA-DR4. IA-2 JM antibodies are non cross reactive (the JM domain differs significantly between IA-2 and IA-2β). Recognition of the JM domain leaves more of the PTP region of the IA-2 molecule available for digestion in the antigen processing compartments of the B cell, possibly leading to enhanced processing of IA-2, and presentation of more peptides to T cells leading to a more rapid immune response (Naserke, Ziegler et al. 1998).

1.5.7 IA-2 Epitopes:
The majority of recognized epitopes to date fall within the intracellular domain (601-979) of the IA-2 molecule, although there is one report of reactivity to an epitope in the extracellular domain (Morran, Casu et al. 2010). These regions of reactivity can be further divided into three major regions of reactivity: the JM domain, central region and C-terminus. Of these, the JM domain is known to appear earliest in disease, and according to results of a study in Finland, presence of IA-2 JM autoantibodies are significantly associated with pre diabetes (Hoppu, Harkonen et al. 2004) and progression to disease, however, these antibodies commonly disappear on follow up (Krause, Chmiel et al. 2012). Reactivity to epitopes within the JM domain is considered to be restricted to a maximum of three epitope regions, identified using monoclonal mouse antibodies and deletion mutants, followed up with a more in-depth study using TRX-chimeric molecules, representing linear sections of the JM domain (Lampasona, Bearzatto et al. 1996; Bearzatto, Naserke et al. 2002; Bearzatto, Lampasona et al. 2003).

The central region epitopes were uncovered at first detection of IA-2 antibodies- only a proportion of patient sera bound both the truncated ICA512E transcript of IA-2, which is missing the JM domain (Payton, Hawkes et al. 1995), which lead to studies using deletion mutants to determine major

Epitopes have also been mapped to the PTP region of the molecule, (Naserke, Ziegler et al. 1998; Kawasaki, Sera et al. 2002; Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009). Reactivity to epitopes within the PTP region of the molecule is the most common, and the most diverse response. There are conformational epitopes within the PTP domain in the C terminus of the molecule (931–979) and within the central region (795–889) (Lampasona, Bearzatto et al. 1996; Bonifacio, Lampasona et al. 1998; Bearzatto, Lampasona et al. 2003). There are several residues which are considered to be of major importance for binding of serum antibodies to PTP domain epitopes:

1. 862, 822
2. 876, 877, 878 and 880 (part of the Tyrosine phosphatase WPD loop, critical for substrate catalysis in active molecules)
3. Two cysteine residues (c909, c945) have been identified as important players in recognition of epitopes within the PTP region of IA-2 (Seissler, Schott et al. 2000; Elvers, Geoghegan et al. 2013).
4. 858, 836, and 799 (Dromey, Weenink et al. 2004), of which the glutamate 836 residue is necessary for binding of many central region antibodies.

1.5.8 Epitope Spreading
Epitope spreading occurs when an immune response to one epitope on an antigen leads to reactivity to epitopes within the same antigen, and closely related antigens (IA-2JM, IA-2PTP, IA-2β). Epitopes may not be similar in structure or sequence, but may be recognized as a result of processing and presentation by antigen presenting cells, recognizing the whole antigen via a primary epitope. This leads to expansion of T cell and antibody repertoires, and a subsequent expansion of antigenic targets recognized by the immune system. Analysis of autoantibodies from both Type 1 Diabetic patients and first degree relatives demonstrates 98% of patients who produce antibodies to IA-2β also recognize IA-2, but 10% of subjects with IA-2 antibodies did not cross react with IA-2β, suggesting that subject first develop antibodies to IA-2, then IA-2β (Kawasaki, Yu et al. 1998) via intramolecular spreading. Yu et al (Yu, Rewers et al. 1996) demonstrated that autoantibodies to islet autoantigens appear sequentially, with insulin autoantibodies usually appearing first, followed by intermolecular spreading to other islet antigens.
1.6 Determining conformational epitope structure:
There are many commonly used techniques to determine protein structure; only Nuclear Magnetic resonance (NMR) and X-ray crystallography can determine the structure of a conformational epitope, although crystallization itself may change the conformation of the epitope. Methods used to determine linear epitope structures, or analyze the contribution of one or more amino acid residues to a given epitope include both site directed mutagenesis and phage display. More structural analysis of epitope conformation requires the use of NMR, which in contrast with other methods can provide a map of the whole protein, and protein complexes, but is severely limited in that unlike other analytical techniques (Mass spectrometry (MS), X-ray diffraction, circular dichroism spectroscopy, Fourier transform IR spectroscopy), NMR requires milligram amounts of material for data generation.

1.6.1 Site directed mutagenesis:
Involves single or multiple substitutions of specific amino acids, to change the epitope recognized at a particular position; mutated proteins can be expressed and antibody binding of the mutants compared with that of the wild type to determine if there is any effect on binding. This technique is expensive, time consuming, requires care in interpretation, as mutagenesis may introduce structural changes in the protein and unwanted disturbance in protein conformation; but is overall an excellent method for determining the location of B cell epitopes (Sivalingam and Shepherd 2012).

1.6.2 Phage display
Phage display permits the detection of continuous peptide epitopes, and allows the detection of some conformational epitopes (Irving, Pan et al. 2001). Phage display requires a bacteriophage vector, with the target gene inserted. The host bacteria are infected by bacteriophage, and the target protein is displayed on the surface as part of the capsid protein (Smith 1985). Therefore, a large library of different peptide epitopes can be displayed by phage, and tested against antibodies known to bind target protein, in order to determine the epitope recognized. The applications of phage display to larger conformational epitopes are limited, and the structure of a protein as a peptide may not be representative of that protein in its native form, however, the technique may allow definition of ‘mimotopes’ that can then be mapped onto the structure of the antigen, if the 3D structure is known.

1.7 Nuclear Magnetic Resonance
Nuclear Magnetic resonance (NMR) is a technique which makes use of a radio frequency signal given off by protons in a molecule as the molecules relax from magnetization. Proton, electrons and
neutrons within the nucleus of an atom all have charge. When charge moves, it gives rise to a magnetic field. The chemical environment surrounding a nucleus will influence the frequency given off by the nucleus, generating a $^1\text{H}$ proton spectrum. The frequency generated by protons is recorded in p.p.m; some nuclei will line up parallel to the external field, and others anti parallel. To rotate the magnetic field, a radio frequency is pulsed across the molecule, which forces the nuclei to drop out of alignment. The pulse used in NMR is called an excitation pulse: this is usually 0.1-10 kW and for duration of 1-100 microseconds. Parts of the molecule lying transverse to the magnetic field change orientation around the field direction. The frequency of the precession is proportional to the strength of the magnetic field. The strength of the radio frequency pulse used to excite the molecule is determined by the Larmor frequency of the labeled isotope(s). Each pulse used can only excite one isotope.

NMR works by measuring a signal generated by applying an external energy field in the form of a second magnetic field which causes a change in state of the nuclei with spin, from the lower energy level to a higher energy level. The change in energy state can be recorded as a radio frequency. When the external energy field is removed, the nuclei return to their base energy level, emitting another radio frequency signal, which can also be recorded. The radio frequencies can be recorded in a number of different ways, to generate different NMR spectra. Only nuclei with spin will generate an NMR signal. Most nuclei have spin, however, those with an even mass nucleus (even numbers of protons and neutrons) have a spin which cancels out, and will therefore equal zero. Nuclei with an even mass nuclei but an odd number of protons and neutrons will have integral spin and those with an odd mass nuclei (odd number of nucleons) have fractional spin.

To detect the rotation of the magnetized nuclei, a receiver coil is used to detect changes in the radiofrequency emitted by the magnetized nuclei. Once the excitation is finished, the nuclei relax back into their equilibrium state. The route which a nucleus takes to return to equilibrium is known as a relaxation curve. The relaxation curve is complicated, and usually defined in two parts: $T_1$, spin-lattice relaxation, where the longitudinal parts of the molecule return to equilibrium; $T_1$ is $1/R$, where $R$ is the rate of relaxation, and $T_2$, spin-spin relaxation, the way in which the transverse part of the molecule decays exponentially to equilibrium. $T_2$ is always smaller than or equal to $T_1$. $T_2$ is defined by $T_2=\sim 1/\Delta\nu^{1/2}$, where $\Delta\nu^{1/2}$ is the line width of the peak generated at half height.

### 1.8 Using NMR to map an antibody epitope

The standard method of protein epitope identification in Type 1 diabetes has to date used site directed mutagenesis or phage display. As epitopes are often conformation dependent, the information provided by both techniques can be limited. Recent improvements in bioinformatic
algorithms allow fast prediction of possible epitopes, but the accuracy of these predictions is not perfect. A three dimensional model of antibody antigen interactions performed using X-ray crystallography has been commonly used to date; however, crystal structures are difficult to obtain. To uncover the structure of an epitope using NMR, a recording is made of labeled protein antigen alone, followed by a recording of the antibody/antigen complex. Chemical shift perturbations in the $^{15}$N labeled spectrum can be attributed to changes in the chemical environment of residues affected by antibody binding.

**1.8.1 Labeling for NMR:**

**1.8.1.1 $^{15}$N**

$^{15}$N is a necessary label, because the naturally occurring $^{14}$N cannot be observed. A $^{15}$N label is one of the most straightforward labels used in NMR. $^{15}$N labels can be used to generate standard solution-NMR $^1$H $^{15}$NHSQC spectra used to verify protein folding. $^{15}$N-labelled protein can be used to measure dynamic NMR spectra, such as N-H residual dipolar couplings. For smaller proteins, those less than 150 amino acids, a $^{15}$N label can be used to generate a NOESY spectrum, to assign $^1$H and $^{15}$N backbone resonances (McIntosh, Wand et al. 1990).

**1.8.1.2 $^{2}$H$^{15}$N**

Double labeling of proteins for NMR in this study was performed using $^2$H$^{15}$N. The NH groups are exchangeable, which means that they will back-exchange to $^1$H when the protein is purified in a normal aqueous solution. Deuteration of the non exchangeable $^2$H is used to improve the spectrum generated for larger proteins, which relax quickly. Deuterating the protein removes most $^1$H atoms which decreases the speed of relaxation, allowing better resolution of signals.

**1.8.2 Spectra used for analysis of IA-2 PTP:**

**1.8.2.1 $^1$H $^{15}$N HSQC:**

Heteronuclear Single Quantum Correlation is a two dimensional shift correlation, which correlates a single bond from a proton to another, non hydrogen nucleus. The measurements record the transfer of magnetization from the proton to the other nucleus, usually $^{15}$N, giving a peak for each covalently bonded $^1$H-$^{15}$N group. In most cases, a $^{15}$N-HSQC spectrum shows a single peak for each backbone amide proton (one peak represents one peptide bond, apart from any peptide bonds involving prolines). Also seen are single peaks for each indole NH sidechain of tryptophan residues, double peaks for the sidechain amide groups of asparagine and glutamine residues, along with guanidinium and amine groups of lysine.
1.8.2.2 Chemical shift Perturbations following protein-protein interactions:
In the $^2$H$^{15}$N HSQC experiment, each individual NH sidechain should generate an individual peak. The frequency of each peak depends on the local chemical environment of the residue. The environment of a residue will change upon ligand binding, and this causes chemical shift perturbations. In order to determine the location of a binding site, spectra of bound and free protein can be compared, to identify peaks which move due to a change in environment. Knowledge of the backbone assignment of the protein allows determination of which peaks have changed, thereby enabling mapping of the binding site, and allowing definition of the antibody epitope.

1.8.2.3 $^1$H $^{15}$N TROSY:
Transverse relaxation optimized spectroscopy is used for larger molecules. The length of relaxation time for a large molecule is decreased, which leads to an increased peak width (defined by the line width at half height) and an increase in the signal to noise ratio making the peaks broader and more likely to overlap (Riek, Wider et al. 1999). Using TROSY on larger, deuterated molecules gives rise to one sharper peak for each amide in the spectrum (Wider and Wuthrich 1999). The disadvantage of TROSY is that it usually requires higher magnetic fields. The spectrum generated in TROSY is similar to that generated by an HSQC experiment (Bardelli, Livoti et al. 2015). A TROSY spectrum is necessary for a molecule as large as IA-2 PTP, which would otherwise relax too rapidly to generate a clear spectrum.

1.9 Hypothesis:
The hypothesis underlying this study is that there are epitopes on autoantigens recognized by B cells that are common to many patients with type 1 diabetes. An understanding of these common epitopes could be exploited for the specific depletion of these B cells in order to prevent disease.

1.10 Aims and Justification:
The presence of islet autoantibodies in Type 1 diabetic patients was reported in 1974 by Bottazzo et al (Bottazzo, Florin-Christensen et al. 1974). The presence of self-reactive antibodies is indicative of a dysregulated immune system. This dysregulation results in a loss of tolerance to multiple β-cell antigens, leading to β-cell destruction and overt disease onset. An understanding of the self-epitopes recognized is therefore critical for use of antigen-specific immunotherapy for the prevention of disease.

The development of antibodies to one of the major islet autoantigens (GAD65, Insulin, IA-2, ZnT8 and Tspan-7) is indicative of a loss of B cell self-tolerance, and can be detected many years prior to disease onset. The role of the B cell as an antigen presenting cell in the early stages of disease has
been recognized, but remains as yet undefined. Studying specific epitopes recognized by B cells in early disease and any association of these epitopes with HLA will help to clarify the role of B cells in early disease onset.

The role of the B cell as an antigen presenting cell in diabetes has been well defined by several groups in the mouse (Serreze, Gaskins et al. 1993; Serreze, Fleming et al. 1998; Hu, Rodriguez-Pinto et al. 2007; Serreze, Chapman et al. 2011). Selection of autoantigen by B cells is less well defined: this study uses the IA-2 autoantigen as a model to define specific epitopes and determine associations with high risk HLA and develop antibody fragments directed to this antigen. Antibodies to IA-2 can be detected in up to 75% of recent onset patients. Both B and T cells capable of recognizing IA-2 epitopes have been isolated from peripheral blood of recent onset patients. The project takes advantage of the presence of several monoclonal anti-IA-2 antibodies, of both human and mouse origin, as well as access to a cohort of recent onset Type 1 diabetic patients (within six months of diagnosis) to characterize epitopes for autoantibodies to a major target of the autoimmune response in the disease. Epitope characterization of proteins can be performed using several different methods:

1. Peptide array scanning uses a library of short non overlapping peptides on a chip, which represent the whole molecule. This allows mapping of regions of reactivity of an antibody but does not allow detection of conformational epitopes (Poetz, Ostendorp et al. 2005).

2. Phage display allows analysis of large numbers of peptides, but as this method recognizes linear epitopes relating such information to conformational epitopes may be problematic. (Rowley, O’Connor et al. 2004).

3. Site directed mutagenesis: introducing single amino acid changes to candidate areas. This approach is both slow and time consuming, allowing only a small number of candidate amino acids to be analyzed. It cannot give any information about the structure of a particular epitope.

4. X-ray crystallography allows visualization of antibody protein interactions - crystallization can be difficult for some proteins.

5. Nuclear magnetic resonance (NMR) allows in solution mapping of antibody binding to specific residues. NMR is faster than X-ray crystallography, and will reveal the structure of conformational epitopes; whilst providing a more complete map of epitopes structure than that provided by phage display, peptide mapping or site-directed mutagenesis.
Many recent studies have focused on the role of the T cell as an effector cell in diabetes. However, B cell depletion is an effective method of slowing disease (Pescovitz, Greenbaum et al. 2009; Yu, Herold et al. 2011; Pescovitz, Greenbaum et al. 2014) progression in humans, and preventing disease onset in mice (Henry, Kendall et al. 2012). Therefore, this project will focus on the role of the B cell in diabetes. The study will attempt to:

1. Synthesize and express functional antibody fragments cloned from EBV-transformed, monoclonal B cell lines, known to secrete antibody recognizing the IA-2 antigen.
2. Isolate and clone variable heavy and light chain sequences from single B cells recognizing IA-2 from peripheral blood samples from recently diagnosed patients. These sequences can then be cloned to produce functional antibody fragments.
3. Determine whether IgG variable heavy (VH) genes can be amplified from formalin fixed, paraffin embedded (FFPE) pancreatic tissue from recent onset type 1 diabetic patients, in collaboration with Anne Clark in Oxford, Alan Foulis in Glasgow, Noel Morgan and Sarah Richardson in Exeter.
4. Further investigate binding of epitopes in the central region of the IA-2 molecule, using solution NMR to assess the feasibility of this technique in clarifying the location of PTP domain epitopes.
5. Investigate epitopes recognized by IA-2 reactive patients for a less well defined antigenic region of the IA-2 molecule: the juxtamembrane (JM) domain, using site directed mutagenesis of a radiolabeled construct. Recognition of the JM domain is associated with expression of HLA-DR4; further clarification of epitopes within this region may lead to a stronger association with HLA types.
Chapter 2  Cloning and expression of recombinant antibodies from human and mouse B cell lines secreting antibodies to IA-2:

2.1 Introduction:

B cells bind antigen via a high affinity receptor- the B cell receptor (BCR), which has the same antigen specificity as its secreted form, the antibody. B cells endocytose and process antibody-bound antigen prior to presenting peptides to T cells. The ability of antibodies to influence antigen processing and affect which epitopes of which antigens are presented on MHC class II molecules to T cells could very well determine disease outcome. To date very few B cell clones have been isolated from type 1 diabetic patients. Two of these with specificity for the autoantigen, IA-2, were available for use in this study: 96/3 and DS329. Both 96/3 and DS329 recognize a well characterized region in the PTP domain of IA-2, with amino acid E836 playing a key role in epitope binding (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009). This region is also implicated in T cell reactivity in diabetes, suggesting a link between antibody binding and antigen presentation to T cells (McLaughlin, Gulati et al. 2014). A third monoclonal antibody, 76F, of mouse origin, but recognizing an epitope in the juxtamembrane domain of IA-2 that is similar to an epitope in human diabetes, was also used (Piquer, Valera et al. 2006).

Development of an efficient method to clone and express functional antibody fragments at high concentrations will permit more efficient characterization of epitopes recognized by antibodies and their role in regulating the T cell response. Therefore, this study set out to extract messenger RNA from frozen aliquots of EBV transformed human B cells, in order to amplify and clone V-region genes and create recombinant antibody fragments for use in future studies. Following the generation of the first monoclonal antibodies, by hybridoma fusion, or Epstein Barr virus transformation, the generation of recombinant antibodies and antibody fragments permits more detailed study of binding and epitope recognition of antibodies (Hust, Meyer et al. 2011; Reichert 2011).

Although the Fab fragment is the recombinant antibody fragment most closely related to natural antibody, the single chain fragment variable (scFv) retains similar antigen binding capabilities, but was specifically designed for use in phage display. Both Fabs and scFvs retain the whole antigen binding site, however, the advantage of the scFv is that both heavy and light chain variable domains are attached by a linker sequence, allowing for more efficient flexible bacterial expression (Holliger and Hudson 2005; Jeong and Rani 2011). The lower molecular weight of the scFv makes it more suitable for many experimental and clinical imaging applications, including structural studies by NMR (Yuasa, Koyama et al. 2013), one of the objectives of this PhD project. A whole IgG molecule consists...
of four chains, two heavy and two light chains. Each light chain has one variable and one constant domain, each heavy chain has one variable and several constant regions. The V-region contains three hypervariable loops, which determine antigen binding, whilst being supported by conserved framework sections. An scFv is a ~25 kDa heterodimer consisting of variable heavy and light regions, joined by a peptide linker, in this case a multimer composed of glycine and serine. The scFv is the smallest antibody fragment containing both parts (VH and VL) of the antibody binding site (Andris-Widhopf, Steinberger et al. 2011).

The variable light and heavy domains of an antibody are assembled into an scFv fragment by overlap extension PCR (Kreberger, Bornhauser et al. 1997). Primers are designed to include an appropriate linker, of 9-15 amino acids in length; in this case, we opted for a 14mer linker. This chapter describes a reliable method for amplification of variable region genes, from cell lines known to produce antibody. In this part of the study, wherever sequence was known, we used sequence-specific primers for reverse transcription to prevent mis-priming and generation of out of frame templates. Resulting sequences were cloned into a vector for expression in E coli.
The expression vector, pAK19 (Carter, Kelley et al. 1992), shown in Figure 2-1, was used to express antibody fragments. The pAK19 vector confers both tetracycline and ampicillin resistance. The vector uses a system where protein expression is induced by low-phosphate concentrations and contains an OmpA sequence, which produces an N-terminal peptide targeting the *E. coli* outer membrane protein A to the Sec translocase, which translocates the OmpA tagged protein into the periplasm. Here the OmpA signal peptide is removed by a signal peptidase (Binet, Letoffe et al. 1997). Periplasmic expression of protein prevents accumulation in insoluble inclusion bodies (Pines and Inouye 1999). The location of EcoRI/HindIII sites allow the removal of the whole insert including OmpA sequence, and use of MluI/HindIII to remove only the insert after OmpA.

To verify immunoreactivity expressed protein products were tested in competition with monoclonal antibodies for antigen binding, and if possible against antibodies in patients’ sera to determine...
specificity of binding. Two scFvs, 96/3 and 76F were later cloned into a second expression vector, pFLAG CTS, to allow direct binding of antigen, trapping scFv-antigen complexes using a FLAG tag.

2.2 Aims:
The purpose of the research described in this chapter was to clone and express functional antibody fragments from monoclonal B cell lines, capable of producing antibodies reactive to the IA-2 antigen. Two of these antibodies, 96/3 and DS329 were isolated from recent onset type 1 diabetic patients. There is evidence that these antibodies are representative of patient serum reactivity (Weenink, Lo et al. 2009). The 96/3 monoclonal antibody was generated by EBV transformation of an IA-2 reactive B cell from a Type 1 diabetic patient (Kolm-Litty, Berlo et al. 2000). Reactivity of this antibody has been well characterized: the antibody has been previously shown to compete for IA-2 binding with antibodies from patient sera, and with two other monoclonal antibodies, M13 and DS329 both of which recognize similar epitope regions within the IA-2 molecule. Phage display assays identified a consensus binding sequence of Asn-x-Glu-x-x-(aromatic)-x-x-Gly for the 96/s antibody (Dromey, Weenink et al. 2004), follow up assays using site directed mutagenesis allowed localization of the binding site of the antibody to A858, E836, and W799 (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009). DS329 is a monoclonal B cell line generated at Kings College London, by EBV transformation of IA-2 reactive B cells from a type 1 diabetic patient, followed by screening of transformed clones, obtained by limiting dilution, for IA-2 autoantibodies. Unfortunately, the EBV line was unstable and viable proliferating cells were eventually lost, leaving only the cloning of V-regions from residual cells as a method of recovering the antibody. The specificity of the antibody is directed to the central region of IA-2, specifically amino acids LHVE (831-836)-KNV (857-859) (Weenink, Lo et al. 2009). Substitution of these amino acids was also shown to inhibit binding of serum autoantibodies from IA-2 antibody positive type 1 diabetic patients, inferring that this antibody recognized an epitope common to a large number of patients. In addition to two human monoclonal antibodies, this study also uses a mouse monoclonal antibody to the juxtamembrane (JM) domain of the IA-2 protein. The mouse monoclonal antibody 76F was characterized by Piquer et al (Piquer, Valera et al. 2006). 76F is capable of specifically recognizing IA-2 (76F), not IA-2 β. The epitope for 76F was defined as being residues 626-630 of the IA-2 molecule, represented by amino acids (FEYQD) which are found in the JM region of human and mouse IA-2 but not in IA-2β. This region is similar to the “JM2” autoantibody epitope recognized by type 1 diabetic patients (Bearzatto, Lampasona et al. 2003).
2.3 Materials and methods:

2.3.1 Synthesis, PCR amplification and cloning of IgG V regions from IA-2 specific B cell lines

The 96/3 rFab was synthesized by Genscript using the previously published sequence (Kolm-Litty, Berlo et al. 2000). The scFv fragment of 96/3 was generated by PCR amplification of 96/3 DNA to introduce both a linker sequence and new restriction site, in order to subclone the assembled antibody fragment into the pAK19 vector. The DS329 rFab and scFv fragments, and 76F scFv fragments were generated using the following method:

Total cellular RNA was isolated from cryopreserved cells using an Invitrogen Purelink mini RNA kit. Cells were thawed at 37°C, spun down to remove growth media, and washed in PBS. Cell pellets were then lysed, using lysis buffer with β-mercaptoethanol and washed through a minicolumn. RNA was eluted in water, and quantified using a Nanodrop device (Thermo Scientific). Concentrations of RNA recovered was between 70-500 ng/μl for <600 0000 cells.

**Figure 2-2:** scheme demonstrating method for amplifying DS 329 and 76F immunoglobulin V region genes.

RT-PCR was performed on 76F and DS329 mRNA, using a Promega access RT PCR kit, with mixed leader/hinge primer pools for DS329 (shown in Table 2-4, 1 and 2), and 76F (shown in Table 2-4 5). A table detailing the RT-PCR conditions is shown below in Table 2-1.
Table 2-1: Components for OneStep RT-PCR reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (Final Concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP Mix (containing 10mM of each dNTP)</td>
<td>2.0µl (400µM)</td>
</tr>
<tr>
<td>Forward and reverse primers</td>
<td>0.5µl (0.6µM)</td>
</tr>
<tr>
<td>QIAGEN OneStep RT-PCR Enzyme Mix</td>
<td>2.0µl</td>
</tr>
<tr>
<td>Extracted RNA</td>
<td>2µl/reaction</td>
</tr>
<tr>
<td>5x QIAGEN OneStep RT-PCR Buffer</td>
<td>10.0µl (1x)</td>
</tr>
<tr>
<td>RNAsin (Promega) RNase inhibitor</td>
<td>5 units/reaction</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>To a final volume of 50µl</td>
</tr>
</tbody>
</table>

The reverse transcription reaction was set up as shown in Table 2-1, and performed at 50 °C for 30 minutes, followed by 40 cycles of PCR. Products were analyzed by gel electrophoresis, 10µl of each reaction was run in a 1% agarose gel, bands representing IgG V region products of 200-300bp were excised and purified, using a Bio-Rad Freeze and Squeeze kit. Bands were diced and placed in a column within a dolphin tube, and frozen for 5 minutes. Tubes were then spun for 5 minutes and DNA in the flow through quantified using a Nanodrop device. A follow up PCR was assembled using specific primer pairs, shown in Table 2-4 1 and 2, or for 76F Table 2-4 5. The reaction was assembled as detailed in Table 2-2, and cycled for 40 cycles, as shown in Table 2-3.

Table 2-2: Components for GoTaq G2 PCR reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (Final Concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq® Reaction Buffer 5X Colorless</td>
<td>10µl (1X)(contains 1.5mM MgCl2)</td>
</tr>
<tr>
<td>Forward and reverse primer mix</td>
<td>5µl each mix, (1.0µM)</td>
</tr>
<tr>
<td>GoTaq® G2 DNA Polymerase (5u/µl)</td>
<td>0.25µl (1.25u)</td>
</tr>
<tr>
<td>PCR Nucleotide Mix, 10mM each dNTP</td>
<td>1µl  (0.2mM)</td>
</tr>
<tr>
<td>RT-PCR reaction</td>
<td>2µl</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>To a final volume of 50µl</td>
</tr>
</tbody>
</table>

Following generation of a PCR product/cDNA, individual nested heavy and light chain PCR reactions were set up, using primer pairs shown in Table 2-4 3 and 4. As each individual primer had a different annealing temperature, determination of optimal annealing temperatures for amplification of heavy, lambda and kappa chains was required. This was performed using a gradient PCR, which identified an overall optimal annealing temperature of 55°C. Cycling conditions for PCR are shown in Table 2-3.
### Table 2-3: cycling conditions for V region gene PCR

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>94°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>72°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>72°C</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

Repeat x 50 cycles

1. **Forward Leader:** Forward human IgG leader region primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHldr1</td>
<td>CCATGGACTGGACCTGGAGG</td>
</tr>
<tr>
<td>VHldr2</td>
<td>ATGGACATACTTTGCTCCAC</td>
</tr>
<tr>
<td>VHldr3</td>
<td>CCATGGAGTTKGGGCTGAGC</td>
</tr>
<tr>
<td>VHldr4</td>
<td>ATGAAACACCTTGGTTCTTT</td>
</tr>
<tr>
<td>VHldr5</td>
<td>ATGGGGTCAACGCCCATCCTT</td>
</tr>
<tr>
<td>VHldr6</td>
<td>ATGTCTGTCTCCTCCCTCAT</td>
</tr>
<tr>
<td>VKldr1</td>
<td>CTCAGCTCCTGGGGCTCC</td>
</tr>
<tr>
<td>VKldr2</td>
<td>CTGCTCAGCTCCTGGGCC</td>
</tr>
<tr>
<td>VKldr3</td>
<td>GGAARCCCAGCDCCAGC</td>
</tr>
<tr>
<td>VKldr4</td>
<td>CTCTCTGCTCTGGATCTCTG</td>
</tr>
<tr>
<td>VKldr5</td>
<td>GGGTCCCAAGTCCACCTCCTT</td>
</tr>
<tr>
<td>VLldr1</td>
<td>CCTCCTCCTCACCCTCCT</td>
</tr>
<tr>
<td>VLldr2</td>
<td>CTCCCTAAGGCGCACAG</td>
</tr>
<tr>
<td>VLldr3</td>
<td>ATGGCCTGGAYCCCTCCTCCTSC</td>
</tr>
</tbody>
</table>
### 2. Reverse hinge: Reverse Primers Human IgG hinge region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgGHinge</td>
<td>GG(AGT)CTGGGCAYSRTGGGCAY</td>
</tr>
<tr>
<td>CL2</td>
<td>CGCCG(TCTAGA)ACTATGAACATTCTCTAG</td>
</tr>
<tr>
<td>CK1Z</td>
<td>GGCCTGG(TCTAGA)ACTAACACTCTCCTGTTGAAGCCTTTGGACGCGTACCA</td>
</tr>
<tr>
<td>CH1</td>
<td>GCATGT(ACTAGT)TTTGTCAAGATTTGGG</td>
</tr>
</tbody>
</table>

### 3. Forward coding region: Human IgG Coding region primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VK1a</td>
<td>RACATCCAGATGACCAG</td>
</tr>
<tr>
<td>VK1b</td>
<td>GACCATCCAGTTGACCAG</td>
</tr>
<tr>
<td>Vk1c</td>
<td>GCCATCCRGATGACCAG</td>
</tr>
<tr>
<td>VK1d</td>
<td>GTCTCTGGATGACCAG</td>
</tr>
<tr>
<td>VK2a</td>
<td>GATATTTGATGACCAG</td>
</tr>
<tr>
<td>VK2b</td>
<td>GATRTTGATGACCTAG</td>
</tr>
<tr>
<td>VK3a</td>
<td>GAAATTGTGTGACCAG</td>
</tr>
<tr>
<td>VK3b</td>
<td>GAAATAGTGATGACCAG</td>
</tr>
<tr>
<td>VK3c</td>
<td>GAAATTGTAAATGACACAG</td>
</tr>
<tr>
<td>VK4a</td>
<td>GACATCGTGATGACCAG</td>
</tr>
<tr>
<td>VK5a</td>
<td>GAAACGACACTACGCAG</td>
</tr>
<tr>
<td>VK6a</td>
<td>GAAATTGTGTGACTACAG</td>
</tr>
<tr>
<td>VK6b</td>
<td>GATGTGTGTGACACAG</td>
</tr>
<tr>
<td>VL1a</td>
<td>CAGCTGTGCTGACTACAG</td>
</tr>
<tr>
<td>VL1b</td>
<td>CAGCTGTGCTGACTACAG</td>
</tr>
<tr>
<td>VL1c</td>
<td>CAGCTGTGCTGACGACG</td>
</tr>
<tr>
<td>VL2</td>
<td>CAGCTGTGCTGACTACAG</td>
</tr>
<tr>
<td>VL3a</td>
<td>TCCTATGWGCTGACTACAG</td>
</tr>
<tr>
<td>VL3b</td>
<td>TCCTATGAGCTGACACG</td>
</tr>
<tr>
<td>VL3c</td>
<td>CAGCTGTGCTGACTCAG</td>
</tr>
<tr>
<td>VL3d</td>
<td>TCCTATGAGCTGACTCA</td>
</tr>
<tr>
<td>VL4</td>
<td>CAGCTGTGCTGACTCA</td>
</tr>
<tr>
<td>VL5</td>
<td>CAGCTGTGCTGACTCA</td>
</tr>
<tr>
<td>VL6</td>
<td>AATTTATGTGACTCA</td>
</tr>
</tbody>
</table>
### 4. Reverse nest: Reverse primers: IgG coding region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lnest</td>
<td>GC(TCTAGA)ACTAATGCGTGACCTGGCAGCTGT</td>
</tr>
<tr>
<td>Knest</td>
<td>GC(TCTAGA)ACTAATGGGTGACTTCGCAGGCGTAGAC</td>
</tr>
<tr>
<td>Hnest</td>
<td>GG(ACTAGT)TCTTGTCCACCTTGGGTCTG</td>
</tr>
</tbody>
</table>

### 5. Primers for mouse IgG

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ MsVHE</td>
<td>GGGATTCGAGTTGCAGCTTGGCAGGTCTGG</td>
</tr>
<tr>
<td>3’ Cy2b outer</td>
<td>GGAAGGCTGCACACTGCTGGAC</td>
</tr>
<tr>
<td>3’ Cy2c inner</td>
<td>ACTCAAGGGAAGTAGCCCTTGAC</td>
</tr>
<tr>
<td>PCR 5’ L-Vk_3</td>
<td>TGCTGCTGCTCTGGGTTCCAG</td>
</tr>
<tr>
<td>5’ L-Vk_4</td>
<td>ATTTCAAGGTCTTGGCTCTAATC</td>
</tr>
<tr>
<td>5’ L-Vk_5</td>
<td>TTTTGCTTTTCTGGATTYCAG</td>
</tr>
<tr>
<td>5’ L-Vk_6</td>
<td>TCGTTTCTSTGGTGTCTG</td>
</tr>
<tr>
<td>5’ L-Vk_6,8,9</td>
<td>ATGGATACAGRGCCWGGT</td>
</tr>
<tr>
<td>5’ L-Vk_14</td>
<td>TCTTGTGCTCTGGTGYCCAG</td>
</tr>
<tr>
<td>5’ L-Vk_19</td>
<td>CAGTCCCTGGGCTCTTTGTGCTC</td>
</tr>
</tbody>
</table>

---

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL7</td>
<td>CAGRCTGTGGTGACTCAG</td>
</tr>
<tr>
<td>VL8</td>
<td>CAGACTGTGGTGACCCAG</td>
</tr>
<tr>
<td>VL4_9</td>
<td>VCGGACTGTGCTGACTCAG</td>
</tr>
<tr>
<td>VL10</td>
<td>CAGGCAGGGGCTGACTCAG</td>
</tr>
<tr>
<td>VH1a</td>
<td>CAGGTCACCTGCTGGCAG</td>
</tr>
<tr>
<td>VH1b</td>
<td>CAGGTCAGCTTTGGTGAG</td>
</tr>
<tr>
<td>VH1c</td>
<td>SAGGTCCAGCTTGGTACAG</td>
</tr>
<tr>
<td>VH1d</td>
<td>CARATGCAGCTTGGCAG</td>
</tr>
<tr>
<td>VH2a</td>
<td>CAGATCACCTTGAAGGAG</td>
</tr>
<tr>
<td>VH2b</td>
<td>CAGGTCACCTTGGTGAG</td>
</tr>
<tr>
<td>VH3a</td>
<td>GARGTTGCAGCTTGGTACAG</td>
</tr>
<tr>
<td>VH3b</td>
<td>CAGAGTGCAGCTTGGTACAG</td>
</tr>
<tr>
<td>VH3c</td>
<td>GAGGTCAGCTTGGTGAG</td>
</tr>
<tr>
<td>VH4a</td>
<td>CAGSTGAGCCTGAGGAG</td>
</tr>
<tr>
<td>VH4b</td>
<td>CAGGTCAGCTACAGCAG</td>
</tr>
<tr>
<td>VH5a</td>
<td>GARGTGCACCTGCTGGCAG</td>
</tr>
<tr>
<td>VH6a</td>
<td>CAGGTCAGCTGAGGAG</td>
</tr>
<tr>
<td>VH7a</td>
<td>CAGGTSCAGCTTGGCAGA</td>
</tr>
</tbody>
</table>

---

5. Primers for mouse IgG

1. **VL**:
   - VL7: CAGRCTGTGGTGACTCAG
   - VL8: CAGACTGTGGTGACCCAG
   - VL4-9: VCGGACTGTGCTGACTCAG
   - VL10: CAGGCAGGGGCTGACTCAG
   - VH1a: CAGGTCACCTGCTGGCAG
   - VH1b: CAGGTCAGCTTTGGTGAG
   - VH1c: SAGGTCCAGCTTGGTACAG
   - VH1d: CARATGCAGCTTGGCAG
   - VH2a: CAGATCACCTTGAAGGAG
   - VH2b: CAGGTCACCTTGGTGAG
   - VH3a: GARGTTGCAGCTTGGTACAG
   - VH3b: CAGAGTGCAGCTTGGTACAG
   - VH3c: GAGGTCAGCTTGGTGAG
   - VH4a: CAGSTGAGCCTGAGGAG
   - VH4b: CAGGTCAGCTACAGCAG
   - VH5a: GARGTGCACCTGCTGGCAG
   - VH6a: CAGGTCAGCTGAGGAG
   - VH7a: CAGGTSCAGCTTGGCAGA

---

4. Reverse nest: Reverse primers: IgG coding region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lnest</td>
<td>GC(TCTAGA)ACTAATGCGTGACCTGGCAGCTGT</td>
</tr>
<tr>
<td>Knest</td>
<td>GC(TCTAGA)ACTAATGGGTGACTTCGCAGGCGTAGAC</td>
</tr>
<tr>
<td>Hnest</td>
<td>GG(ACTAGT)TCTTGTCCACCTTGGGTCTG</td>
</tr>
</tbody>
</table>

---

5. Primers for mouse IgG

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ MsVHE</td>
<td>GGGATTCGAGTTGCAGCTTGGCAGGTCTGG</td>
</tr>
<tr>
<td>3’ Cy2b outer</td>
<td>GGAAGGCTGCACACTGCTGGAC</td>
</tr>
<tr>
<td>3’ Cy2c inner</td>
<td>ACTCAAGGGAAGTAGCCCTTGAC</td>
</tr>
<tr>
<td>PCR 5’ L-Vk_3</td>
<td>TGCTGCTGCTCTGGGTTCCAG</td>
</tr>
<tr>
<td>5’ L-Vk_4</td>
<td>ATTTCAAGGTCTTGGCTCTAATC</td>
</tr>
<tr>
<td>5’ L-Vk_5</td>
<td>TTTTGCTTTTCTGGATTYCAG</td>
</tr>
<tr>
<td>5’ L-Vk_6</td>
<td>TCGTTTCTSTGGTGTCTG</td>
</tr>
<tr>
<td>5’ L-Vk_6,8,9</td>
<td>ATGGATACAGRGCCWGGT</td>
</tr>
<tr>
<td>5’ L-Vk_14</td>
<td>TCTTGTGCTCTGGTGYCCAG</td>
</tr>
<tr>
<td>5’ L-Vk_19</td>
<td>CAGTCCCTGGGCTCTTTGTGCTC</td>
</tr>
</tbody>
</table>

2.3.2 Cloning and sequencing of PCR products representing individual antibody V-region transcripts

Purified PCR products were ligated into a sequencing vector, pGEM-T-easy (Promega, UK), using T4 ligase. Reagents for ligation are detailed below in Table 2-5. Reagents were assembled on ice and incubated for 1 hour at room temperature. Ligated plasmids were transformed into chemically competent XL1B cells by incubating 1µl of the ligation reaction with 50µl cells on ice for 30 minutes. Cells were then heat shocked for 45 seconds at 42°C, followed by 2 minutes on ice. Cells were grown up in 500µl NZY+ broth, the spread onto LB Agar- Ampicillin plates, and incubated overnight at 37°C. The following day single colonies were picked into 5ml LB-ampicillin and grown up overnight.

Table 2-5: Reagents for ligation of PCR products in pGEM-T-easy vector

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid Ligation Buffer, T4 DNA Ligase</td>
<td>5µl</td>
</tr>
<tr>
<td>pGEM-T-Easy Vector (50ng)</td>
<td>1µl</td>
</tr>
<tr>
<td>PCR product (150ng)</td>
<td>Xµl</td>
</tr>
<tr>
<td>T4 DNA Ligase (3 Weiss units/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>Deionized water to a final volume of</td>
<td>10µl</td>
</tr>
</tbody>
</table>

2.3.3 Transformation and Preparation of Ligated plasmids

In order to purify DNA from overnight cultures, cells were spun down and plasmids prepared using the Bio-Rad Quantum Prep plasmid prep kit, as shown in Table 2-6. Purified plasmids were digested using EcoRI to verify the presence of inserts and sent for sequencing (Source Bioscience).
Table 2-6: Bio Rad Quantum Prep Mini prep method

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ml overnight culture</td>
<td>spun for 4 minutes 4400 rpm</td>
</tr>
<tr>
<td>Removed supernatant medium, added 200µl cell resuspension solution to pellet,</td>
<td>Resuspended pellet by vortexing</td>
</tr>
<tr>
<td>Added 250µl lysis solution to resuspended cells</td>
<td>capped tube and mixed by inverting 10 times</td>
</tr>
<tr>
<td>Added 250µl neutralization solution</td>
<td>capped tube and mixed by inverting 10 times</td>
</tr>
<tr>
<td>Supernatant transferred to spin filter containing 200µl of mixed matrix</td>
<td>spun for 30 seconds</td>
</tr>
<tr>
<td>Filtrate discarded, 500µl wash buffer added</td>
<td>spun for 30 seconds</td>
</tr>
<tr>
<td>Filtrate discarded</td>
<td>spun for 2 minutes</td>
</tr>
<tr>
<td>Spin filter placed in a sterile 1.5ml centrifuge tube</td>
<td>added 100µl sterile water (preheated to 37°C)</td>
</tr>
<tr>
<td>Incubated for 1 minute</td>
<td>Spun for 1 minute at top speed</td>
</tr>
<tr>
<td>Spin filter discarded</td>
<td>Plasmid DNA stored at -20°C</td>
</tr>
</tbody>
</table>

2.3.4 Overlap Assembly PCR to generate constructs for antibody expression as scFv

![Overlap Assembly PCR diagram](image)

Figure 2-3: Overlap PCR to generate transcript of an scFv fragment. VL and VH were amplified separately, with linker primers containing part of the linker sequence, which overlaps. When DNA purified from these separate reactions is combined in one tube, the complementary ends anneal, and amplification from the ends generates a full sequence.
Having obtained both heavy and light chain sequences, the variable regions were combined to form an scFv sequence. The construct was amplified with primers shown in Table 2-7, first using separate PCR cycles, followed by an overlap PCR, with extended annealing and melting cycles, to encourage overlapping of complimentary regions of the linker sequence. This reaction generated a whole scFv fragment with both variable heavy and light chain regions linked by a Gly$_4$-Ser$_3$ linker (Krepper, Bornhauser et al. 1997).
Table 2-7: Primers used to generate 96/3 scFv; GS primers code for part of the linker sequence, forward primers contain an MluI restriction site, reverse primer has a penta histidine (His) tag and HindIII restriction site.

cDNA generated from rFab cultures was amplified using specific primers shown in Table 2-7. Table 2-8 shows primers used to generate DS329 scFvs, including three forward light chain primers (for three different light chains) and one reverse primer for the constant region of the light chain.

Table 2-8: Primers for amplification of DS329 V region genes in preparation for overlap extension PCR. As for 96/3, GS primers code for part of the linker sequence, forward primers contain an MluI restriction site, reverse primer has a penta His tag and HindIII restriction site.

This was done using a (Gly-Ser)$_3$ linker sequence first added between the VH/ VK DNA, and the joining both sequences using overlap assembly PCR to joining complementary sequences of the linker the 14mer linker used in this study (GGGSGGGSGGGS), which becomes a 15mer linker when including a glycine in the immunoglobulin sequence. A two-step overlap extension PCR was performed to generate the full scFv sequence. Heavy and light chain variable regions were amplified and gel-purified prior to assembly PCR. The linker sequence is generated by using two inner primers which overlap. To run an assembly PCR, 100ng of DNA representing each variable segment was added, alongside the outer primers used during the pre-assembly (light chain forward primer, with restriction site, heavy chain reverse primer with his tag and restriction site). In order to promote overlapping of the linker, a longer (1.5 minutes) annealing phase was used in the PCR reaction. In
order to subclone into the pFLAG-CTS expression vector the 76F scFv was reamplified using primers shown below in Table 2-9, to insert restriction enzyme sites matching those within the target vector (EcoRI/SalI).

Table 2-9: Primers for cloning 76F into the pFLAG-CTS vector incorporating EcoRI and SalI restriction sites.

<table>
<thead>
<tr>
<th>Primer name:</th>
<th>Primer sequence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>76F EcoF</td>
<td>GAATTCACATTGTGATGACACA</td>
</tr>
<tr>
<td>76F Sal1R</td>
<td>GTCGACTGACTGAGGTTCC</td>
</tr>
</tbody>
</table>

2.3.5 **TOPO ligation of 76F scFv**

The 76F scFv was prepared for subcloning into pFLAG CTS using the TOPO vector system (Invitrogen). The purified PCR amplification of the 76F scFv was ligated into the pCRII-TOPO vector by incubating for ten minutes at room temperature, with salt solution. Ligation reactions were then transformed into TOP10 cells, using the method described in 2.3.3.

2.3.6 **Ligation into pAK19 for Expression of rFab/ scFv fragments:**

Antibody fragments were digested out of the sequencing vector, pGEM-T-easy, using Mlu1/ HindIII restriction sites introduced into the construct during assembly PCR. PAK19 was also digested with MluI/HindIII. Cut pAK19 plasmid was dephosphorylated using thermosensitive alkaline phosphatase (TSAP; Promega, UK), and the digested scFv was ligated into the vector overnight. Ligated plasmids were transformed into XL1B cells, and spread on tetracycline/ampicillin plates. Individual colonies were picked, grown overnight in LB/tetracycline, and plasmid DNA was prepared using a Quantum prep Mini Prep plasmid purification kit (Bio-Rad). The concentration of DNA was measured by absorption at 260 nm using a Nanodrop device (Thermo scientific, UK), and the plasmid digested with MluI/HindIII to confirm presence of inserts, and sent for sequencing (Source Bioscience). After sequencing, purified plasmids were transformed into competent Rosetta Gami or BL21 (Novagen) cells for protein expression using a phosphate induction system in minimal media.

2.3.7 **Ligation into pFLAG-CTS for expression**

Antibody fragments were digested out of the sequencing vector, pCRII-TOPO, using EcoRI/SalI restriction sites introduced into the construct by PCR. pFLAG-CTS was also digested with EcoRI/SalI. Digested pFLAG-CTS plasmid was dephosphorylated using thermosensitive alkaline phosphatase (TSAP; Promega, UK), and the digested scFv was ligated into the vector overnight. Ligated plasmids were transformed into XL1B cells, and spread on LB agar/ampicillin plates. Individual colonies were
picked, grown overnight in LB ampicillin, and plasmid DNA was prepared using a Quantum prep Mini Prep plasmid purification kit (Bio-Rad). The concentration of DNA was measured by absorption at 260 nm using a Nanodrop device (Thermo scientific, UK), and the plasmid digested with EcoRI/Sali to confirm presence of inserts, and sent for sequencing (Source Bioscience). After sequencing, purified plasmids were transformed into competent BL21 (Novagen) cells for protein expression using induction by addition of IPTG in LB broth.

2.3.8 Expression of rFab/ scFv fragments using pAK19:

Medium used for expansion of vector-containing cells is a MOPS based minimal medium (Neidhardt, Bloch et al. 1974) with added phosphate. Colonies were grown for 12 hours at 20-25°C in 10ml Luria Broth (LB), and spiked into 500ml of complete MOPS medium. Cultures were then grown at 25°C and optical density at 600nM (O.D.600) monitored for 8-12 hours. Once O.D. reached 0.75, expression was induced. Induction of protein expression is performed by centrifuging cells at 5,000 rcf for 10 minutes, at room temperature, and resuspending in phosphate free MOPS medium (Carter, Kelley et al. 1992). Protein expression was performed for 8 hours at 25°C and pellets were sedimented at 10,000 rcf for 15 minutes at 4°C.

Pellets were frozen and stored at -20°C, and then lysed in extraction buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH7.5), using a polytron, followed by 3 x 10 second bursts of sonication on ice. Lysate was then treated with lysozyme (final concentration of 0.1mg/ml), followed by DNAse treatment (final concentration 160 Units per 5ml lysate) for 10 minutes at room temperature. Lysates were centrifuged twice at 10,000 rcf to remove cellular debris, and cleared lysates passed through a 0.44um filter.

Filtered lysates were bound onto a NiATA agarose gel bed (His Select, Sigma) for 30 minutes at room temperature. His-select resin is capable of binding the penta-his tag on the VH region of the antibody fragment. The gel bed was washed five times with extraction buffer and protein eluted from the gel bed using 10x 1ml fractions of Hepes containing 300mM imidazole. Fractions were analyzed by SDS PAGE electrophoresis to determine which fractions contained protein, then protein rich fractions were dialyzed using Slide-a-Lyzer dialysis cassettes (Peirce, UK), overnight against PBS. Crude extract protein concentration was measured, using the BCA assay (Bicinchoninic Acid test, Pierce, USA) and final yields of desalted protein were determined using a Nanodrop device. Activity of the protein was then tested using a competition radioligand binding assay, described below in 2.3.11.
2.3.9 Expression of scFv fragments using pFLAG CTS

The pFLAG CTS vector generated a fusion protein with a C-terminal FLAG tag. The FLAG tag is a hydrophilic 8-amino acid peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys (DYKDDDDK)), with an enterokinase cleavage site. Expression of scFv fragments in pFLAG-CTS were performed using IPTG induction: 5ml cultures were grown overnight in LB-Ampicillin. The 5ml overnight culture was then added to 1L of LB-Ampicillin, and grown until the culture reached an O.D. of 0.75. 100mM IPTG was added to a final concentration of 1mM, and the culture was maintained at 37°C for 6 hours. Cultures were centrifuged at 10,000 rcf, then pellets lysed using extraction buffer (50mM NaH2PO4, 300mM NaCl, pH7.5) by rolling at room temperature. Lysate was then treated with lysozyme (final concentration of 0.1mg/ml), followed by DNAse treatment (final concentration 160 Units per 5ml lysate) for 10 minutes at room temperature. Lysates were centrifuged twice at 10 000 rcf to remove cellular debris, and cleared lysates passed through a 0.44um filter.

Lysates were added to anti-DYKDDDDK (L5 antibody coated) resin (Biolegend), and rolled at room temperature for 4 hours. Activity of scFv fragments was assessed using luminescence assays, and purity by SDS-PAGE and western blotting.

2.3.10 Assessment of purity of recombinant antibody preparations using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining and Western blotting

Purified extracts were dialyzed overnight into PBS using 10K cutoff dialysis cassettes (Thermo Scientific). After dialysis, gel samples were prepared. Gel samples were prepared by adding 6µl crude extract or eluted fractions to 4µl 4X LDS (Lithium dodecyl sulphate) NuPAGE sample buffer (Invitrogen), 2µl reducing agent (stabilized DTT) which generated a final volume of 12µl. Samples were then heated for 10 minutes at 70°C. 6µl of each sample was run on 12% Bis-Tris SDS PAGE gels (Invitrogen). Gels were run in MES (Invitrogen) running buffer, at 200 V constant voltage for 35-40 minutes. NOVEX sharp pre stained protein standards (Invitrogen) were used as a ladder to determine protein molecular weight.

Once the dye front had migrated to the bottom of the gel, gels were split open: One gel was stained by Coomassie, to check protein purity. Coomassie R-250 was used (1% Coomassie R-250, 10% Acetic acid, 30% Methanol, 60% H2O) and gels were microwaved for 1 minute on full power, prior to staining for 1 hour. Gels were destained overnight, with three changes of destain reagent (10% Acetic acid, 20% Methanol, 70% H2O). A second gel was transferred onto a PVDF membrane after electrophoresis. The membrane was pre-equilibrated in methanol (10 minutes) rinsed, then equilibrated in NuPage transfer buffer (5% Transfer buffer, 10% methanol, 85% H2O, 1ml
antioxidant). The proteins were transferred to the membrane, at 30 V constant voltage for 1 hour. The membrane was then blocked in 10% skimmed milk powder in PBS-T. Membranes were probed overnight with a biotinylated anti-penta-His antibody (Abcam), at a concentration of 1/1000. Poor results were obtained using streptavidin-Horse radish peroxidase (HRP), therefore the proteins were detected using HRP-conjugated anti mouse IgG (Fc specific), then developed using HRP substrate (2ml 2%3-amino-9-ethylcarbazole (AEC) in acetone, 69ml H2O, 2ml 1M sodium acetate pH 5.5, 125µl H2O2). rFab fragments migrate on SDS-PAGE gels at 38Mr, and scFvs at 28Mr.

2.3.11 Radioligand Binding Assays

Direct radioligand binding assays (RBA) were performed by incubating 5µl antibody (diluted in normal human serum) with 5µg rFab/scFv (diluted in immunoprecipitation buffer), with 20µl containing 20,000cpm IA-2ic translate (representing the whole intracellular region of the IA-2 antigen, from amino acids 605-979). The translate was generated using IA-2IC in a psp60polyA vector, transcribed and translated in the presence of 35S methionine using a TNT SP6 Quick-coupled transcription-translation kit (Promega, UK). Incorporation of the radiolabel was measured by washing 1µl spots of translate on 3M paper in 10% TCA at 60°C prior to drying and counting by scintillation counting in Scintisafe fluid. 5µl of serum or monoclonal antibody dilution in serum were added to radiolabeled translates were diluted in immunoprecipitation buffer (IMP) (10mM HEPES, 150mM NaCl (pH 7.4), 10mM benzamidine, and 2% Triton X-100, 0.5 %BSA and 0.5mM methionine, pH 7.4) overnight in a 96 well plate. Negative controls were performed with appropriate volumes of IMP. The negative control used for direct binding assays was an rFab fragment generated from a mouse monoclonal antibody to the thyroid stimulating hormone receptor, 9.1 provided by Dr. P. Banga.

10µl 50% Protein A Sepharose in H2O was added to capture immune complexes (not Fab or scFv) and incubated for 30 minutes at 24°C. Complexes were transferred to a filter plate (NUNC) washed five times with IMP, twice with H2O, and dried. 100ul Microscint 50 was added and plates were counted using a Chameleon scintillation counter (Hidex).

2.3.12 Luminescence Assays

Luminescence assays were used to determine binding of antibodies to IA-2 IC. Direct binding assays were performed using 10µl of scFv conjugated to anti-DYKDDDDK resin (Biolegend), and incubated overnight with 20µl of 100 000 Light Units (LU) sterile filtered (0.2µM) IA-2 IC translate (amino acids 605-979), at 4°C. The translate was generated using IA-2IC in a pTNT- Renilla vector (generously provided by Dr. Lampasona, Milan), transcribed and translated in the presence of 10mM methionine
using a TNT SP6 Quick-coupled transcription-translation kit (Promega, UK). Success of transcription was measured by adding 1µl of translate with 100µl of renilla luciferase substrate.

After incubation of antibody with antigen, immune complexes were washed, five times in PBS-Tween buffer, then washed in H₂O and resuspended for measurement in 100µl Renilla Luciferase substrate (Promega, UK).

To investigate the ability of the scFv fragment to compete for antigen binding with naturally produced antibodies, 5µl of serum or monoclonal antibody dilution in serum were added to IA-2 IC translates, diluted in PBS-T buffer overnight in a 96 well plate. scFv fragments were eluted off anti-FLAG resin using 100mM glycine buffer (pH 3) and neutralized using 1M Tris (pH 8.5). Negative controls were performed with appropriate volumes of Tris-glycine. 10µl 50% Protein A Sepharose in H₂O was added to capture immune complexes (not Fab or scFv) and incubated for 30minutes at 24°C. Complexes were transferred to a filter plate (NUNC) washed five times with 200µl IMP buffer then twice with H₂O. 100ul luciferase substrate (Promega, UK) was added and plates were counted using a Chameleon luminescence counter (Hidex).


2.4 Results

2.4.1 Cloning and expression of rFab and scFv from the IA-2 B cell line, 96/3

DNA sequence for the 96/3 monoclonal antibody was previously published (Kolm-Litty, Berlo et al. 2000), allowing heavy and light chain cDNA of the antibody, with leader sequences to be synthesized commercially (Genscript) for the expression of rFab fragments. The construct was provided cloned into the pUC37 vector. The sequence of the synthesized rFab is shown in Table 2-10.

96/3 VH4VL3 rFab sequence

<table>
<thead>
<tr>
<th>96/3 VH4VL3 rFab sequence</th>
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<tbody>
<tr>
<td>TGTATTTGTAACATGAATCCGAGCTCGGTACCCCGGGATCCTAGACTAGTTAGTGAAGTTTATGAAAAAG</td>
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<td>AATATCGGATTTCCTTTCTGATCTATGTTGCTTTTTCTATATTGCTAAACACGTATGCTCTCTATGTGCTGAC</td>
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</tr>
<tr>
<td>ACCACCAGCAGGGGCATGCG</td>
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Table 2-10: rFab Light and heavy chain sequence of 96/3 monoclonal antibody.

The Fab fragment was removed from the pUC37 vector by restriction digest, and ligated into PAK19 (Carter, Kelley et al. 1992) for expression in *E. coli* as recombinant Fab fragments (rFab) with a hexa-histidine tag. This vector uses a low phosphate induction system to induce expression of plasmid encoded protein, but permits generation of disulphide bonds, necessary for generation of a functional rFab fragment. Growth and expression were carried out in MOPS minimal media (Neidhardt, Bloch et al. 1974), which can slow growth rates, and lessen yield of target protein, but is necessary for induction of protein expression with the pAK19 vector.
2.4.2 Generation of 96/3 ScFv fragments

Both heavy and light chain variable regions were amplified from the 96/3 rFab template (Figure 2-4A). Bands generated migrated at 220-250bp. Light and heavy chain variable region cDNA bands were purified by agarose gel electrophoresis, and an overlap assembly PCR performed as described in the methods, using only light chain forward and heavy chain reverse primers to overlap and join the linker sequence. Linked scFv fragments generated run at 760bp, as seen in Figure 2-4B.

![Figure 2-4: Amplification of heavy and light chains from 96/3 template for generation of scFv fragments A. amplification of VH and VL products from 96/3 rFab template. B. showing overlap PCR product at 760bp, C. showing restriction digest of pGEM-T easy with 96/3 scFv insert (750bp), and PAK19 with rFab insert (1500bp).](image)

The scFv was ligated into pGEM-T-easy and transformed into XL1B cells for sequencing. Colonies were selected, and plasmid DNA extracted for digestion. Plasmids were digested with EcoRI, to
screen for the presence of inserts prior to sequencing. Sequencing of colonies confirmed correct sequences had been generated for colonies 1, 2, 3 and 6. DNA from colonies with inserts was digested using MluI and HindIII (Figure 2-4C), showing 750bp scFv sequences and 3000-5000bp plasmids. The digested scFv fragments were ligated into predigested pAK19, at room temperature, overnight. Ligations were transformed into XL1B cells, and selected on LB-tetracycline agar plates.

**Figure 2-5:** Digest of 96/3 scFv in pAK19 MluI/HindIII. The plasmid in lane 4 does not contain an insert, hence no lower band is visible, and the band in lane 5 runs slightly too high to be the correct product.

Colonies from ligation reactions were picked, grown overnight, and digested with EcoRI/HindIII to verify the presence of inserts. Clones with correct scFv fragment inserts were transformed into BL21 cells for protein expression using low phosphate induction, overnight.

### 2.4.3 Protein Expression

The optimum temperature and timing were determined as room temperature growth for 12 hours, followed by room temperature expression for a further 12 hours. Yields went from 0.158mg/ml to 1.75mg/ml using these conditions, as shown in Table 2-11.

**Table 2-11:** optimization of growth and expression of 96/3 scFv protein

<table>
<thead>
<tr>
<th>Growth/Expression duration (hours)</th>
<th>Yield (mg/ml) per L culture</th>
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</thead>
<tbody>
<tr>
<td>10 hours growth, 4 hours expression</td>
<td>0.153 mg/ml</td>
</tr>
<tr>
<td>10 hours growth, 5 hours expression</td>
<td>0.538 mg/ml</td>
</tr>
<tr>
<td>12 hours growth, 12 hours expression</td>
<td>1.75 mg/ml</td>
</tr>
</tbody>
</table>

Following protein expression, cells were pelleted by centrifugation, lysed with lysozyme, and extracted scFv fragments were purified using NiATA resin (His Select, Sigma, UK) Expression was
verified by SDS-PAGE followed by Coomassie staining. Both Fab and scFv fragments were also immunoblotted to verify presence of His tagged protein products of the expected size using an antibody to the His tag present on the recombinant fragments (anti-penta-His antibody horseradish peroxidase (HRP) conjugate). Both Coomassie stained gels and Western blots can be seen in Figure 2-6.

2.4.4 Testing reactivity of both scFv and rFab fragments to IA-2

Binding activity of the 96/3 rFab was first tested using an RBA, with his tagged rFab fragments linked to His-select beads. Unfortunately, as seen in Figure 2-7, the level of background generated using His-select beads was too high to distinguish between a positive and a negative result, as the negative control rFab 9.1 also binds IA-2 in this assay. Therefore, a decision was made to test His tagged antibodies by measuring inhibition of native antibody.
Figure 2-7: Analysis of rFab antigen reactivity by direct binding assay, using 96/3 rFab bound to his select beads, with an rFab, generated from a mouse monoclonal antibody to the thyroid stimulating hormone receptor, 9.1 as a negative control. For comparison, binding of whole 96/3 antibody and normal human serum (AB+) were also used as controls, indicative of non antigen-specific reactivity of rFab with IA-2IC.

Recombinant antibody fragments were tested for immunoreactivity by the inhibition of binding of $^{35}$S methionine labelled IA-2IC to whole 96/3 antibody. Results, shown in Figure 2-8, show that even at a dilution of 1:100 the recombinant 96/3 Fab fragment is still able to fully compete with antibody for binding to radiolabelled antigen. This suggests that the rFab fragment has immunoreactivity and is suitable for use in determining epitope specificity of antibodies in competition assays.

Figure 2-8: Inhibition of whole antibody binding by addition of purified 96/3 rFab. A maximum of 5µg of rFab in 5µl PBS was added to either pooled normal human serum (AB+) or 96/3 monoclonal antibody. The rFab was also dd at dilutions of 1/500 and 1/1000, and dilution was accounted for by adding 5µl of PBS to control wells. The 96/3 rFab was capable of inhibiting antibody binding at all dilutions.
A similar inhibition experiment, using the scFv fragment of the 96/3 antibody, shows the scFv fragment is also capable of abolishing whole antibody binding, demonstrating that this recombinant fragment also retains antigen binding capability (Figure 2-9). ANOVA analysis of data, with Bonferroni correction, shows a significant inhibition by addition of scFv, p<0.0001.

Figure 2-9: Inhibition of intact 96/3 antibody binding to IA-2IC by addition of recombinant scFv fragments from four different colony preparations. P<0.0001 between negative control (PBS) and each of the different scFv preparations.

2.5 Cloning and expression of rFab and scFv from the IA-2 B cell line, DS329

DS329 is an EBV transformed B cell line, obtained by screening transformed peripheral blood B cells from a type 1 diabetic patient, which produced a monoclonal antibody recognizing the central region epitope of IA-2. The DS329 cell line was unstable, and due to the loss of viable frozen stocks, attempts were made to clone recombinant antibody fragments, using mRNA extracted from frozen cells.

RNA was extracted using the Invitrogen Purelink microRNA kit, and amplified using an Access RT-PCR system (Promega, UK). RT-PCR was followed by nested second PCR reactions, using GoTaq DNA polymerase (Promega, UK).

Products from the nested two step PCR reactions were run in a 1.5% Agarose/TAE gel. This showed that although there was only one heavy chain amplified, from the VH3 family, there were several light chain bands. All bands were gel purified and reamplified using specific nested IgG primer pairs. The products from this reaction, shown in Figure 2-10 were subsequently purified, ligated into pGEM-T-easy and sequenced. Results of sequencing showed one heavy chain, sequence from the VH3c family. Three light chains were identified, and screened against V-base 2: one kappa light
chain, from the VK1 family, and two lambda chains, one VL2, which was functional, and one VL3c, all shown in Table 2-12.

Figure 2-10: Nested follow up PCR on DS329. A. is amplification using pairs of heavy chain primers, B. Kappa primers, and C. Lambda primer pairs. Products of appropriate size were detected for VH3, VK1-3a, 4a,5a and VL2, 3a, 3c and VL7.

Two Fab fragments representing the single cloned heavy chain with each of the light chains generated by RT-PCR of transcripts from DS329 cells: constructs VH3VK1 and VH3VL2, were designed, and synthesized commercially (Genscript); these were provided cloned into the pUC37 vector. The Fab fragments were designed with an Sfi digest site at the beginning of the light chain, and an Xmal site at the end. This allowed a reamplified version of the VL3 nonfunctional chain, with corrected sequence to be digested and ligated in to the vector with the heavy chain, as seen in Figure 2-11.
Figure 2-11: Reamplification of Vl3c using corrected primers (A), Sf1 XMAJ digest of Vl3c PCR product and PAK19 VH3 VL2 (B).

<table>
<thead>
<tr>
<th>DS329: V-region Sequences for rFab</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Light Chain: V-region sequence VK1</strong></td>
</tr>
<tr>
<td>GDFMKKNIALLASMVFVSIA</td>
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<tr>
<td><strong>Light Chain: V-region sequence VL3c</strong></td>
</tr>
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<td>GDFMKKNIALLASMVFVSIA</td>
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</tr>
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</tr>
<tr>
<td>LGTQTYCINFHKSNTKVKRVEPKSDKTSTCGHEHHHHH</td>
</tr>
</tbody>
</table>

Table 2-12: Light and heavy chain section sequences of Fab fragments for DS329. Bold text represents the OmpA sequence, targeting expressed rFab protein to the periplasmic space.
2.5.1 Expression of DS329 rFabs:

The pAK19 vector with heavy and light chains amplified from DS329 RNA (sequences shown in Table 2-12) were transformed into BL21 cells. Expression was performed using the low phosphate induction system in the presence of tetracycline. Expression of His tagged rFab was verified using both Coomassie staining and Western blotting to detect the His tag on the antibody fragment; as can be seen in Figure 2-12, fragments of the correct size were detected in all three lysates. Unfortunately, protein yields were low, and variability in yields of antibody fragments in bacterial expression systems is a common problem.

![Figure 2-12: Coomassie stain of DS329 rFab expression.](image)

Two DS329 rFabs, each with one of the two light chains amplified from the cell line, one VL2, one VK1 light chain, were tested for ability to block binding to IA-2IC of native 96/3 antibody, which recognizes a similar epitope to the DS329 antibody, as shown by our group (McLaughlin, Richardson et al. 2015). rFabs were added to the monoclonal 96/3 antibody, at a concentration of 5µg in 5ul IMP buffer, which has been shown to inhibit binding of monoclonal antibodies in previous assays (96/3 section). The VL3c rFab was also tested, as seen in Figure 2-13. Testing of this fragment showed no
inhibition of central region antibodies. In contrast, the VK1 rFab showed some inhibitory activity against the whole 96/3 antibody as seen in Figure 2-13, with a P=0.0046 (Student’s t-test, against 96/3+PBS), but since yields of recombinant Fab fragments and levels of inhibition were low, with only a decrease of 19% (21929 cpm to 1918 cpm) it was decided to create a smaller more easily expressed scFv fragment.

![Graph showing inhibition of whole 96/3 monoclonal antibody binding to IA-2 IC translate.](image)

Figure 2-13: Inhibition of whole 96/3 monoclonal antibody binding to IA-2 IC translate. Inhibition was tested by adding 5µg rFab in 5µl PBS to 5µl antibody. Inhibition by VK1 rFab is low but significant (p<0.0046).

### 2.5.2 Generation of DS329 scFv fragments

In an attempt to improve yields of recombinant antibody fragments, the three DS329 antibodies were generated as scFv fragments. cDNA was amplified from minipreps of rFab plasmid DNA. As can be seen in Figure 2-14, both heavy and light chains were successfully amplified from cDNA, with clear bands for each chain visible: the heavy chain comes first (VH3) followed by the three light chains, VK1, VL2 and VL3c. The ladder (M) used is the Promega benchtop PCR marker.
Figure 2-14: Pre-overlap PCR of DS329 heavy and light chains. Amplification of VH3, VK1, VL2 and VL3c using primers designed for overlap PCR. Markers shown (from top: 1000bp, 750bp, 500bp, 300bp, 150bp) are Promega benchtop PCR markers.

Once single heavy and light chain PCR products were generated, an overlap PCR was performed, as described for the 96/3 scFv. Fragments were purified on agarose gels, and combined in one PCR reaction, using only the forward primer from the light chain amplification, and the reverse chain from the heavy chain amplification, allowing the overlapping sections of the PCR products to anneal during the reaction. The reaction generated full length scFv products, as can be seen in Figure 2-15 with each scFv running at 750bp.

Figure 2-15: Overlap PCR of DS329 heavy and light chains. VK1, VL2 and VL3c with VH3 generated 3 ScFv products of identical size, 750bp, with the end lane showing molecular weight marker, Promega benchtop PCR markers (from top: 1000bp, 750bp, 500bp, 300bp).

All three scFv products were then ligated into pGEM-T-easy for sequencing. Ligated plasmids were purified and digested to check for the presence of inserts, prior to sequencing. Digests are shown below in Figure 2-16. All scFvs with inserts (VK1-4, VL2 1-4, VL3c 1, 2, 4) were sent for verification by DNA sequencing. The DNA sequences returned, both protein and DNA are shown below in and all were verified as derived from rearranged immunoglobulin gene products. The predicted protein sequences are also shown in Table 2-13.
Figure 2-16: Digests of DS329 scFv products in preparation for subcloning. Showing EcoRI digests of pGEM-T-easy plasmid containing A. VH3VK1 scFv, B. VH3VL2 scFv and C. VH3VL3c scFv. Inserts can be seen in VK1-4, VL2 1-4 and VL3c 1,2 and 4. Markers (M) used are the Promega benchtop 1Kb ladder (A,C) markers showing 10 000bp (top) to 250bp (bottom). Brighter bands represent 1000 and 3000bp. B uses PCR markers (Promega) from top 1000bp, 750bp, 500bp.
DS329 VH3VL2 scFv DNA sequence

```
AACGGGTATGCTAGTCAGTCTGCCCTGACTCAGCCTCCCTCCGCTCCGGGTCTCCTGGACAGTGACCAGTCCCATCTCCTCCGCGTCCGGGTCTCCTGGACAGTGACCATCCTTGGCACTGGAACCAGCAGTGACATTGGAGCTTATAATTATGTGTCCTGGTACCAACAGCACCACCAAGGGAAAGCCCCCAAACTCATAATTTATGAGGTCATAAAGCACCCTAGGGGCTCCTGGATTACACCTTACAGTGAAGACATCTCTCTAGTACCTAGTACATG
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Protein sequence:

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NAYAQSALTQPSASGSPGQSVITSTCGTSGSDDIAGNYVSWSYYQHQPGKAPKLIIYEVIKRPSPGVPDRSGSKGNTASLTOLQAEADYCCSSYAGSNWSVFGGGTGLTVLGGGGS
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DS329 VH3VK1 scFv DNA sequence

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AACGGGTATGCTAGTCAGTCTGCCCTGACTCAGCCTCCCTCCGCTCCGGGTCTCCTGGACAGTGACCAGTCCCATCTCCTCCGCGTCCGGGTCTCCTGGACAGTGACCATCCTTGGCACTGGAACCAGCAGTGACATTGGAGCTTATAATTATGTGTCCTGGTACCAACAGCACCACCAAGGGAAAGCCCCCAAACTCATAATTTATGAGGTCATAAAGCACCCTAGGGGCTCCTGGATTACACCTTACAGTGAAGACATCTCTCTAGTACCTAGTACATG
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Protein sequence:

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104
DS329 VH3VK1 Protein sequence:

NAYADIQMTQSSSASVGDRVTITCRASQGISLAWFQQKPGKPSLGYIYNLWS
GVPSRFSGGSSTGDTFTLTISSLQPDDFATYCYQHYTYPITFGQGTRLEIKRGGSGGGG
SGGGSQVQLVESGGGLVKKPGGSLRSAASSGFTSFDSYMSWIRQAPKGEWVSYILST
SGNTNIHYADSVKGRFTMSRDNAKSSLFLQMNSLRAEDTAVYYCARVRSYAFDSWGQGT
LTVVSSASTKGPSVFPLAPSSKSTHHHHHH

Table 2-13: DNA and predicted protein sequences of DS329 scFvs; Bold text shows primer sequences, underlined text shows linker sequence.

Both VH3Vk1 and VH3VL2 scFvs generated were then digested with MluI and HindIII (Figure 2-17), for subcloning into the expression vector, pAK19. Overnight ligations were performed, and scFv plasmids were transformed into XL1B cells for production of DNA. Colonies were digested with MluI/HindIII to verify the presence of inserts as seen in Figure 2-17. scFvs were then transformed into BL21 cells for protein expression.

![Figure 2-17: assessment of outcome of subcloning of DS329. Digest of VH3VK1, VH3VL2 with MluI/HindIII, showing inserts digested from both plasmids. Markers (M) are the Promega benchtop 1Kb ladder.](image)

2.5.3 Expression of DS329 scFv:

Expression of the scFv was performed using the same methodology as for the rFabs. Levels of expression were higher than previously seen for rFabs, generating up to 1.75mg purified scFv per liter of culture. Protein bands for the scFvs were detected at 28kDa using both Coomassie staining and anti-penta His Western blot, as shown in Figure 2-18, demonstrating expression of His-tagged products of the correct size. Purification of the scFv yields sufficient quantities of protein to perform an inhibition assay to test specificity of the antibody fragments.
2.5.4 Inhibition of native antibody binding using DS329 antibody fragments

As some degree of inhibition had previously been seen using the VH3VK1 scFv, this construct was tested in a blocking assay using 5µM scFv/well to inhibit binding of monoclonal antibodies to 35S-labelled IA-2. The VH3VL2 scFv was also tested for inhibition but showed no effect on binding of monoclonal antibodies or sera. The VH3VL3c scFv was not tested as it showed no sign of inhibition previously.

Figure 2-18: Coomassie stain and western blot of DS329 scFv expression. A. Showing Coomassie of scFv extracts, and B. anti-penta His Western blot.

Figure 2-19: RBA testing inhibition of monoclonal antibody binding by addition of excess DS329 VH3VK1 scFv. 2-way ANOVA with Bonferroni post test shows significant inhibition of DS329 (P<0.01) and R2B2 (P<0.001).
As seen in Figure 2-19, addition of 5uM DS329 VK1 scFv is capable of fully inhibiting binding of DS329 whole antibody to ³⁵S labeled IA-2IC translate. It is also capable of inhibiting binding of other antibodies, M13, and 96/3, which recognize a similar epitope region, but not monoclonal mouse antibody 76F. However it does partially inhibit a polyclonal rabbit antibody, R2B2, which recognizes epitopes predominantly within the JM domain of IA-2.

2.6 Cloning and expression of scFv from the IA-2 juxtamembrane domain specific mouse antibody-secreting B cell line, 76F

2.6.1 RT-PCR amplification of heavy and light chain cDNA from the hybridoma cell line, 76F

mRNA was isolated (as described in the section) from cryopreserved 76F cells, a monoclonal mouse B cell hybridoma line, which expresses an antibody to the JM domain of the IA-2 molecule. This cell line recognizes the JM2 epitope (621-640), the FEYQD motif spanning JM 626–630 was previously identified as the minimal epitope (Piquer, Valera et al. 2006), which is commonly recognized by patient sera (Richardson, McLaughlin et al. 2016).

2.6.2 76F PCR:

Mouse IgG primers (Tiller, Busse et al. 2009) shown in Table 2-4, were used in combination with 50 cycles of RT-PCR, using the QIAGEN OneStep RT-PCR kit, amplifying both VH and VK IgG products. 76F is known to secrete antibody of the IgG2 Kappa isotype, therefore only primers for the kappa light chain amplification were used. RT-PCR produced several bands, possibly because the mouse primers are degenerate and annealing temperatures are relatively low, in order to maximize the possibility of sequence retrieval. RT-PCR products were run in a 1.5% agarose gel. The expected size of the products was 250-300bp. Two V kappa primers produced bands of the expected height, VK3 and VK14. A 300bp heavy chain was also amplified in an independent reaction (shown in Figure 2-20).

![Figure 2-20: 76f RT-PCR. Lanes 3, 14 show bands at 260 bp and VH shows a significant band at ~300kb. Marker is Promega benchtop PCR markers.](image)

Both heavy and light chain bands were ligated into pGEM-T-easy for sequencing. DNA Sequencing
produced one heavy chain sequence and one light chain sequence: the light chain sequence amplified was a nonfunctional gene product, which is present within the SP2 cell line used to form the hybridoma (Leahy, Rule et al. 1988; Piquer, Valera et al. 2006). This cell line is known to express a nonfunctional endogenous V kappa transcript, which encodes a stop codon at position 105 in the CDR3 region (Carroll, Mendel et al. 1988). This aberrant V kappa sequence was present in such abundance that amplification of the true sequence of the 76F kappa light chain was masked.

### 2.6.3 Amplification of the 76F light chain

As an alternative approach to amplify the correct kappa transcript for 76F, PCR products representing the aberrant V kappa chain would need to be distinguished for removal. In order to do this, a specific primer was designed, with the aim of generating a truncated transcript of the aberrant V kappa chain as shown in Figure 2-21, which would then run at a lower molecular weight in a DNA gel, permitting detection of the true 76F light chain transcript within the full length products.

![Diagram showing RT-PCR and primer specificity](image)

**Figure 2-21:** Method for reducing quantity of full length AbVK transcripts produced in RT-PCR, using an AbVK specific primer, added at a high concentration to compete with other Vkappa primers for binding of AbVK RNA.

An RT-PCR program was set up incorporating 30 minutes of RT, followed by 30 cycles of PCR with an annealing temperature of 52°C. This system is optimized for the Qiagen Qiaquick RT-PCR kit. After 30 minutes at 50°C, a pause was programmed to allow the AbVKappa primer to be added (at 200pMol concentration) to all V kappa wells of the plate, as shown in Figure 2-21. In an attempt to block amplification of the AbVK sequence, a primer specific for the AbVK sequence, which generates a
shorter, truncated sequence, was added to a molar excess. As this primer bound the AbVK sequence specifically, would favor amplification of the truncated AbVK sequence, preferentially amplifying AbVK mRNA, leaving other primers free to bind 76F mRNA.

RT-PCR showed two sets of primers which amplified longer products than the AbVK primer (Figure 2-22, VK3, and VK14). The VK12 primers produced longer length products, however there were several products at higher molecular weight suggesting this may not have been specific amplification. All primers produced a truncated 100bp AbVK product, as expected, but there was no amplification of a functional lambda chain. When sequenced, both VK3 and VK14 primers produced the full AbVK sequence.

In order to recover PCR products representing the 76F light chain lacking the aberrant Vkappa transcript from the SP2 cell hybridoma, the upper, full length bands were gel purified using the Promega wizard kit. These were then digested with Alu1 or Rsa1 and analyzed in a 1% agarose gel. Only full length bands were excised and purified. These transcripts were then amplified in a second round of PCR, using semi nested V kappa primers. This second round of PCR yielded a full length band, which was then ligated into pGEM-T-easy and sequenced. With addition of the specific AbVK primer, digestion of resulting PCR products with RSA1/Alu1 and subsequent re-amplification of the digested PCR reaction with nested primers (JK01-04) one full length V kappa sequence out of eight possible clones created (seen in Figure 2-23). The full length sequence came from cloned JK02 19. AbVK sequence (protein and DNA) is shown below in Table 2-14:
AbVK DNA sequence: Stop codon marked in bold.

GACATTGTGCTGACACAGTCTCCTTATTAGCTGTATCTCTGGGGCAGAGGGCCACCATCTCATAACAGG
GCCAGCACAAGTGCAGTACATCTGGGTATAGTTATATGCACGGCAAACAAAACAGGGCAGCAGCCACC
CAGACTCCTCATCTATTTGTATACCAACCTAGAATCTGGGGTTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGG
GACAGACTTTCACCCCTACACATCCATCTGTTGGAGAGGAGGAGATGTCTGCAACCTATTACTGTGACGACATTA
GGGAGCTTACACGTTGGAGGGGACAAGCTGGAAATAAAC

Protein Sequence:
DIVLTQSPASLVGQRATISYRASKSVSTSGYSYMHWNQQKPQPRLIYLVSNLGVPARFSGSGSTDF
TLNIHPVEEADAATYCYCQHIRELTREGGPSWK*N

Table 2-14: AbVK sequences, protein and DNA, bold text shows stop codon.

Figure 2-23: Follow up PCr of 76F; performed on RSAI/AluI digested RT-PCR products, generating expected bands of 250bp for several VK-JK02 primer combinations.

2.6.4 Assembly PCR:
VK02-19 and 76F VH transcripts were amplified using overlapping linker sequence primers, and a reverse heavy chain primer, which also encodes a His tag for future purification. In order to create a scFv fragment, both heavy and light chains were joined together by the linker sequence. A first round of PCR was performed using complementary primers to add part of the linker sequence to the heavy chain, and part of the linker sequence to the light chain. Both chains amplified and produced a 200kd product, as shown in Figure 2-24.

Figure 2-24: Pre-assembly PCR of 76F heavy and light chains. The reaction produced products for both heavy and light chains. Assembly PCR generates large quantities of VH gene sequence, along with an upper band, which represents the entire scFv sequence.

Following the first round of PCR, both VH and VK products were gel purified and reamplified. The
second round of PCR uses only the end primers, VK forward and VH reverse, to overlap and link PCR products together in one scFv fragment. The successful amplification of the 76F product is shown in Figure 2-24. The correct band for the scFv product is the upper band (700kb). The start of the heavy chain contains some sequence similarity to the start of the light chain, allowing some mispriming to occur, which produces the lower 250Kd band seen in Figure 2-24. The 700 kb product was ligated into pGEM-T-easy and sequenced: Sequence is shown in Table 2-15.

<table>
<thead>
<tr>
<th>76F scFv sequence: primers in bold, linker sequence underlined.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AACGCGTATGCTGACATTGATGACACAGACTCCA</td>
</tr>
<tr>
<td>TATTTAGATTGGTCACCTGCAGAAAGCGCCAGTCTCTCCAAAGCTCTGATCATCAAAGTTC</td>
</tr>
<tr>
<td>TCCAAACGATTITCCTGCGTCCACAGACATTGCAGTTTCAGTGGCAGTGGGATAGGGACAGACAGAGTC</td>
</tr>
<tr>
<td>ACCTCAAGATACATAGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAGGT</td>
</tr>
<tr>
<td>TCACATGTTCGCTACGGCTTGTCGGCTCAGTGGGACAGTGGGAAATAAAACGTAC</td>
</tr>
<tr>
<td>GGTGGCGGCTGGCCGGGCTGGTGGGTCGTGGCTGGCAGAGGGTACAGGAGTGCAGCTGAGAGTCT</td>
</tr>
<tr>
<td>GGGGGAGACTTAGTGGAAGCCCTGGAGGTCCCTGAGAACTCTCTGTTCAGCTCCTGGAAGTC</td>
</tr>
<tr>
<td>ACTTTCTCTAATAGCTGTGCTTGGTCTGGCTCGACAGACAGACAGACTGAGTGGG</td>
</tr>
<tr>
<td>GTCGCAACCATGGATAGAAGGGTGCTTTCAAGGT</td>
</tr>
<tr>
<td>TTCACCACCTCTCAGAGACAGAATGTCAGGAAACCTGATCTGCAAATGAGCAGTCTGAAG</td>
</tr>
<tr>
<td>TCTGCTGACAGCCATGATTACTGTACAGGCGACCCCACGCTATGCTAGACTAC</td>
</tr>
<tr>
<td>TGCGTCAAGAAGAACCTCAGTCAAGTGCTCCTACACCACACACCACACACACACTGAAAGCTTGGA</td>
</tr>
</tbody>
</table>

Protein Sequence:

| NAYADIVMTQPTLSPVSLGDQASIQRSQSSIVHRNGNTYLWYLQKAGQSPKLIYKV |
| SKRFPGVDPDGSGLGTDFTLKISRVEAELGVYCFQGHVPLTFGAGTKEIKRTVGGGSGGSGGGGE |
| VQLQESGGDLVKPGGSLKSLCSASGVTFINYGMSVWQTPQKRLW |
| VATIDSSYTYYPSAKGRTFISRDVNRKTLQQMSSLKADTAMYYCTGHPPDAMDY |
| WQGQGTVSSHHHHH |

Table 2-15: Protein and DNA sequences for 76F. Bold text highlights primer regions, underline shows linker.
2.6.5 Assembly of the 76F scFv in pAK19 for protein expression

Figure 2-25: Digest of 76F scFv and expression vector, in preparation for subcloning. Gel shows undigested pAK19 DS329, MluI/HindIII digested pAK19 DS329, undigested PAK19 96/3, MluI/HindIII digested pAK19 96/3, undigested pGEM-T-easy 76F, MluI/HindIII digested pGEM-T-easy 76F.

DNA representing the 76F ScFv was recovered from the pGEM-T-easy cloning vector using MluI and HindIII restriction enzymes. The pAK19 vector, with the 96/3 scFv insert, was digested under the same conditions, and treated with thermo sensitive alkaline phosphatase (TSAP) to prevent vector religation. The scFv insert was ligated overnight into pAK19, transformed into BL21 E.coli, and plated onto tetracycline selective plates. Colonies which grew were spiked into an overnight 5ml culture containing Luria broth and tetracycline. Plasmids were extracted using the Quantum Prep miniprep plasmid purification kit, and digested with MluI to verify the presence of an insert. pAK19 is a low copy number vector, so recovering sufficient plasmid DNA from this vector for sequencing proved difficult.

2.6.6 Expression of 76F scFv

Figure 2-26: Coomassie stain of 76F expression. Gel shows faint band for 76F in elution 3. Note low levels of expression, and low levels of protein in lysate. Marker, from top shows 40kDa then 30kDa.

Colonies containing an insert were grown in LB-Ampicillin. Cultures were grown overnight, transferred into MOPS minimal medium and grown to O.D. 0.75. At this point, cultures were spun
down, and resuspended in phosphate free MOPS medium, to induce protein expression. After 5 hours of expression, cultures were pelleted. Cells were then lysed and protein purified using Ni-NTA resin (His select, Sigma, UK). Presence of protein in eluates was verified using Coomassie staining, as seen in Figure 2-26.

Blocking experiments were performed by Stefan Williams, using desalted, concentrated protein, tested in a blocking assay using 5µM scFv/well to inhibit binding of monoclonal antibodies to 35S-labelled IA-2. These experiments confirmed the scFv was immunoreactive, (Figure 2-27), showing significant inhibition of both a rabbit polyclonal antibody and the native 76F antibody (both p<0.001).

![Figure 2-27: RBA of 76F scFv inhibition of 76F whole antibody. Inhibiton of pooled human serum (AB+), 76F whole Ab, rabbit polyclonal anti IA-2 (R2B2) and the 96/3 central region reactive monoclonal antibody (performed by Stefan Williams a BSc. Student, under my supervision.).](image)

### 2.6.7 Improving Expression of 76F scFv for direct binding assays

To improve expression levels, the 76FscFv was reamplified from the pAK19 vector template, using primers with EcoRI and Sall restriction sites, to allow sub cloning into the pFLAG-CTS expression vector.

![Figure 2-28:PCR amplification of 76F scFV incorporating EcoRI/Sall restriction sites for subcloning into pFLAG-CTS. Product is seen at 730bp. (Markers are Promega benchtop PCR markers).](image)
The amplified 76F scFv product is shown in Figure 2-28. Products were ligated into the pCRII-TOPO vector and transformed into TOP10 cells. Colonies were subsequently picked and expanded, and plasmids were purified and sent for sequencing. One plasmid with the desired scFv sequence was then digested with EcoRI and SalI, alongside the pFLAG-CTS vector. Digested products can be seen in Figure 2-29, below.

Figure 2-29: Digest of 76F scFv in preparation for subcloning. EcoRI/SalI digest of 76F scFv in pCRII TOPO, showing 3 digests of 76F scFv and digested pFLAG CTS for ligation. 76FscFV is 750 bp, markers used are Promega benchtop markers.

The scFv DNA was then ligated into the pFLAG-CTS vector overnight, and transformed into XL1B cells for plasmid purification. Single colonies were grown overnight and plasmids purified. Plasmids were digested with EcoRI and SalI to confirm the presence of an insert, as shown in Figure 2-30. A plasmid containing the insert was selected for expression and transformed into BL21 cells for expression. Expression was carried out in a 1L volume, induction of expression was performed at OD750, using 1mM IPTG.

Figure 2-30: Digest to determine outcome of subcloning of 76F scFv. EcoRI/SalI digest of 76F scFv in pFLAG-CTS, showing digested scFv at 750bp. Markers used are Promega benchtop PCR markers

Cells were spun down and protein purified on anti-FLAG resin, followed by elution of purified protein using 100mM glycine buffer (pH 3) and neutralization in 1M Tris (pH 8.5). The presence of protein in eluates was assessed by SDS page western blot; Mr25K protein was seen in lysate and eluted from anti-FLAG beads, as seen in Figure 2-31, using both colorimetric detection and chemiluminescence.
As the colorimetric detection method is less sensitive, only the large amount of protein in the lysate can be visualized, however the chemiluminescent blot shows protein in lysate and elutions using SDS.

2.6.8 Luminescence Assays of IA-2 binding

Using two renilla luciferase tagged IA-2IC constructs, the ability of the 76F scFv to bind IA-2IC protein was assessed. 76F reactivity was also tested against renilla luciferase. The 76F scFv was able to bind both IA-2IC constructs at a significantly greater levels than luciferase, as can be seen in Figure 2-32.
Figure 2-32: Direct binding assay of 76F scFv. Binding of IA-2IC by the 76F scFv construct, and binding of anti-FLAG beads alone. Significantly more light units were generated when 76F scFv was incubated with IA-2-mono (p=0.0004) and IA-2-tandem (p<0.00001) than luciferase, or anti-FLAG beads with no antibody.


2.7 Discussion:

This section develops a method for amplification of human and mouse immunoglobulin V-region gene sequences, which can be used to generate functional recombinant antibody fragments from monoclonal B cell lines and may be adapted for other sources of antibody transcripts.

Functional antibody fragments were cloned and expressed for all three B cell lines: both rFab and scFv fragments from the 96/3 cell line were capable of competing with whole antibody for the central region epitope. Only the scFv fragment of DS329 was fully efficient at competing for binding sites, possibly due to the complexity of disulphide bonding required for functional rFab fragments; which may be a limiting factor in the quantity of functional protein produced in this system. As scFv fragments have a linker sequence and require no disulphide bonds, they may express more functional fragments than the complex rFab molecule.

Expression levels of protein are construct-dependent; despite many attempts to increase expression levels, the 76F construct did not express at high enough levels to continue with His-tagged expression. As tests against monoclonal antibodies showed that a functional product was being produced, the fragment was subcloned into a different vector, allowing growth in LB, and improved yields of protein, which was assessed using both direct binding assays.

The 96/3 and DS329 constructs efficiently inhibited their own native parent antibody, and other central region monoclonal antibodies, whilst the 76F construct was capable of inhibiting several different monoclonal antibodies binding to the JM domain of IA-2. Inhibition of binding by antibody fragments shows that binding efficiency can be retained in a recombinant system. Unfortunately, the NiATA agarose beads are not specific enough to allow for direct binding assays: tests with an irrelevant mouse rFab to a thyroid antigen showed high levels of immunoprecipitation of IA-2 in direct binding assays, suggesting the radiolabeled antigen was non-specifically binding to the NiATA agarose resin, preventing direct binding assays using this system.

Following the generation of inhibitory rFab and scFv molecules in this study, the 96/3 scFv has been sub-cloned into the pFLAG-CTS (Sigma) vector by Dr. Kerry McLaughlin, for generation of fragments that could be used for direct binding. The FLAG tag is an epitope based tag, allowing more specific purification of tagged protein. Anti-FLAG resin was shown to successfully bind FLAG tagged scFv, which was capable of binding radiolabeled IA-2.

To date, hybridoma generation and EBV transformation have been the methods most commonly to clone human autoantibodies from Type 1 diabetic patients for detailed analysis of the B cell
response in disease. The number of antibodies generated by these methods are few, partly due to low rates of transformation (up to 3%) and poor stability of EBV transformed cells. Successful cloning of immunoglobulin genes is dependent on identification of matching heavy and light gene pairs from individual B cells (Larrick, Danielsson et al. 1989). The use of molecular techniques also permits the generation of detailed sequence information for variable and constant regions of the heavy and light chains: isotype usage in humans is extremely variable (Tiller, Meffre et al. 2008), requiring the use of multiple primers to ensure adequate coverage of the repertoire. There is, however, a predominance of VH3 usage in the periphery. The development of a method to amplify V-region genes from B cell lines will permit the generation of recombinant antibody fragments from multiple sources: single B cells, mouse hybridoma lines, and EBV transformed cell lines.

Bacterial expression is the most widely used method for generation of antibody fragments in large concentrations, although successful expression of a reactive fragment can be problematic (Gasser and Mattanovich 2007; Zarei, Vaziri et al. 2014). Antibody fragments can be created in several different expression systems (mammalian, yeast and bacterial), and there are many variables contributing to the success of bacterial expression, including protein size, folding, solubility, and stability. Lower levels of expression of some fragments may indicate a lack of suitability for bacterial expression systems (Knappik and Pluckthun 1995). The use of periplasmic OmpA tags in this system should prevent direction of protein into inclusion bodies, but may not preclude the production of insoluble protein. Efficient production of some antibody constructs may only be possible in a mammalian system.

To date, antibody fragments have been used for not only therapeutics (Nelson and Reichert 2009) but also for immunodetection, as chaperones to aid crystallization (Griffin and Lawson 2011) and for protein purification (Hunte and Michel 2002). The popularity of these fragments has increased with improvements in expression: since antibody fragments retain their antigen binding properties and can be produced relatively cheaply in bacteria, which are easy to manipulate and cultivate.

Full length monoclonal antibodies as well as antibody fragments represent one of the most important and valuable classes of biopharmaceuticals generated to date. Unfortunately, due to the low levels of surface glycosylation performed in bacterial cells, whole antibody molecules are still generally produced using mammalian expression systems (Dodev, Karagiannis et al. 2014). These are both more expensive to maintain, and express lower levels of protein. Antibody fragments, which are not glycosylated but retain antigen binding properties, can also be produced in bacteria, but the optimization of fragment format (rFab scFv, nanobody) is essential to the successful generation of a functional product.
This study demonstrates that functional scFv fragments can be generated from human monoclonal antibodies; the methods used in this study can be transferred for use with other templates.
Chapter 3  Generation of antibody fragments from single IA-2 reactive B cells

3.1  Introduction:

Prior to the use of single-cell expression cloning, generation of human autoantibodies from type 1 diabetic patients for study have been generated by immortalization with Epstein Barr virus, or by fusion with hybridomas (Kolm-Litty, Berlo et al. 2000; Ananieva-Jordanova, Evans et al. 2005; Piquer, Valera et al. 2006; Lanzavecchia, Corti et al. 2007; Tiller, Meffre et al. 2008; Tiller, Busse et al. 2009). Both of these techniques have limitations in clonal stability and levels of IgG produced. Alternatively, phage display permits random pairing of heavy and light chains to select for antibodies of particular antigen specificity, but this approach is unlikely to select for the natural autoantibodies important for an understanding of the molecular recognition of autoantigen in disease (McCafferty, Griffiths et al. 1990).

Advances in technology now permit the use of single-cell reverse transcription PCR (RT-PCR) to isolate immunoglobulin heavy and light chain variable gene segments from single B cells sorted by flow cytometry (Tiller, Meffre et al. 2008; Tiller, Busse et al. 2009). Variable region genes can then be cloned and expressed in prokaryotic cell lines. This permits the production of functional antibody fragments from any B cell that can be identified using flow cytometry (Wardemann and Nussenzweig 2007). These techniques have now been used by many groups to study antibodies in autoimmunity and disease (Tiller, Meffre et al. 2008; Duty, Szodoray et al. 2009; Meffre 2011). Coronella et al (Coronella, Telleman et al. 2000) developed a method for amplification of IgG sequences, using single cells to generate correct pairing of heavy and light chains.

Catani et al (Catani, Walther et al. 2015) report generating 863 islet antigen reactive antibodies from peripheral blood and pancreatic lymph nodes, by RT-PCR, in conjunction with mammalian expression of whole immunoglobulin molecules. Of the 863 antibodies generated only two were generated that were reactive to an islet autoantigen. Both recognized IA-2.

Using a technique similar to the Coronella group, this study uses single flow cytometry sorted antigen reactive B cells, in combination with antigen capture, to ensure amplification of antigen specific IgG only. Amplification of functional heavy and light chain pairs with correct VDJ rearrangements is usually achieved using PCR amplification of mRNA taken from peripheral blood lymphocytes. Using this method, functional pairings of light and heavy chains can be achieved.
As described in Chapter 2, amplifying immunoglobulin V-region genes, in order to create functional antibody fragments has previously been used to generate both Fab and scFv fragments from monoclonal EBV transformed B cells and hybridomas (Chapter 2). The ability to select single IA-2 positive B cells from type 1 diabetic patient samples permits amplification of heavy and light chain variable region of IgG from these cells. Products can then be reamplified and expressed as antibody fragments, which can be tested against whole antibody in blocking assays to determine specificity of antibody fragments generated. Using a bacterial expression method, with cloned variable region genes would generate stable clones of antigen.

Five monoclonal antibodies from human B cells have been generated to the PTP domain of IA-2. 96/3 (Kolm-Litty, Berlo et al. 2000), DS329 (Weenink, Lo et al. 2009), M13 (Ananieva-Jordanova, Evans et al. 2005), A5 and C6 (Catani, Walther et al. 2015). All of these antibodies recognize a similar region of the IA-2 molecule (Catani, Walther et al. 2015; McLaughlin, Richardson et al. 2015). Generation of a human monoclonal antibody fragment recognizing other epitopes in the IA-2 molecule, expressed at high levels in E.coli would permit the characterization of other responses. Developing a method for higher throughput creation of recombinant antibody fragments from human B cells would permit a broader based analysis of human antibody specificities (Tiller, Meffre et al. 2008). Expression levels of bacterial systems are traditionally viewed as higher than those of mammalian systems, although recent reports demonstrate sustained high levels of expression of whole antibody using the pVITRO vector (Dodev, Karagiannis et al. 2014; Blanco-Toribio, Alvarez-Cienfuegos et al. 2015).

3.2 Aims:
The aims of this study were to clone and express antibody fragments from single IA-2 reactive B cells, isolated from type 1 diabetic patients. Fragment reactivity was then tested using direct binding assays.

In order to clone antibody fragments from single B cells, B cells were isolated from peripheral blood mononuclear cells (PBMCs) from whole blood and antigen-specific memory B cells by labeling with fluorescence tagged IA-2 and anti-CD27 followed by fluorescence-activated cell sorting (FACS) into single wells of 96-well plates. Cells were stored, and IgG variable region transcripts reverse transcribed, followed by a two-step nested PCR amplification. Products were sequenced and sequences analyzed against the V region germline database (V-Base2) and the Genbank database. scFv fragments were generated using an overlap PCR system, and products ligated into an appropriate expression vector.
3.3 Methods:

3.3.1 Patient samples for B cell purification and amplification and cloning of recombinant autoantibodies

Blood samples were obtained with consent from recent onset type 1 diabetic patients in Yorkshire and London. Samples were sent to London by next day delivery, and processed the following day. Total PBMCs were separated using a Lymphoprep gradient, and B cells were purified using a magnetic bead system from Miltenyi (Miltenyi B cell isolation kit II (human)). B cells were frozen using the CTL-cryo-ABC system, while serum antibody phenotypes were determined, using radioligand binding assays performed as described in Chapter 2 (2.3.11).

3.3.2 Selection of high IA-2 autoantibody positive patients for antigen specific B cell purification

B cells were isolated from blood samples from patients determined to be strongly positive for autoantibodies to the antigen of interest. Serum samples from patients were tested for serum antibody reactivity against three major autoantibody targets in Type 1 diabetes: GAD65, Znt8 and IA-2. All samples which tested positive for IA-2 autoantibodies using a construct representing the intracellular domain of the protein, amino acids 605–979, were further tested against three truncated IA-2 transcripts:

1. The JM transcript, representing the juxtamembrane domain (605–693)
2. A shorter construct representing the central region with a c-terminal deletion (amino acids 643–937)
3. The PTP transcript, representing the central region epitope, and incorporating a c-terminal deletion (amino acids 643–979).

Samples were categorized according to which sub-specificities of autoantibodies were detected in RBA, and high-titre IA-2+/JM+ sera were preferred for B cell sorting.

3.3.3 B cell sorting

All FACS sorting and B cell isolation was performed by Dr. Kerry McLaughlin. In preparation for studies on IgG V-region amplification, autoantigen specific B cells in samples were identified by binding of a fluorescence-labeled IA-2 construct to surface IgG that act as antigen receptors. GST (Glutathione S-transferase)-tagged IA-2, bound to FITC (Fluorescein) conjugated antibody to GST, allows FACS sorting of single, IA-2 reactive B cells.
Blood samples were separated by centrifugation on a Lymphoprep gradient for 30 minutes at 2000 rpm in an Eppendorf bench top centrifuge. Memory B cells were then magnetically separated using a Miltenyi memory B cell isolation kit, selecting CD19+/CD27+ cells. Cells were washed in warm PBS, counted, and pelleted at 1400 at 2000 rpm for 10 minutes. Memory B cells and memory B cell depleted PBMCs were cryopreserved at -80°C in a Mr. Frosty, using a CTL-Cryo ABC (Immunospot, Germany) serum free freezing kit, prior to long term storage in liquid nitrogen.

Cells were thawed and sorted on a FACS Calibur, gating for IA-2 (GST tagged, labeled with FITC anti GST) and anti CD27 (PE) CD27, which is a memory marker for B cells. CD27 expression promotes differentiation from memory cell to plasma cell (Agematsu, Hokibara et al. 2000). FACS plots for both antibody positive and negative patients were generated and compared to ensure specificity.

Single cells were sorted into well of a PCR plate containing 10µl Qiagen Buffer EB, spun briefly and snap frozen on dry ice, prior to long term storage at -80°C.

3.3.4 Extraction of mRNA from B cell lines.

mRNA was extracted from cryopreserved B cells derived from a B cell line, using an Invitrogen Purelink mini RNA kit, as described in Chapter 2). mRNA was eluted in water, and quantified using a Nanodrop device (Thermo Scientific) by quantifying wave lengths: 230 nm for background absorption, 260 nm (nucleic acids), 280 nm (proteins), and 320 nm (contaminants and background absorption). The OD260/280 ratio is used as indicator for nucleic acid purity. Initial yield was 37.5 ng/µl. A 1/10000 dilution of mRNA was amplified as described below.

3.3.5 RT PCR of single B cells

mRNA was amplified from individual cells using a Qiagen OneStep RT-PCR kit, and a mix of primers (shown in Table 2-4 1 and 2). Plates containing single sorted cells were thawed on ice and spun briefly (30 seconds) to ensure all cells were in the bottom of wells. In this study, the Qiagen OneStep RT-PCR kit was used in conjunction with a mix of heavy and light chain primers. This was followed by two semi nested PCR reactions. Amplification was performed using a mix of degenerate primers adapted from Sblattero and Bradbury (Sblattero and Bradbury 1998), shown in Chapter 2, Table 2-4. A master mix of one-step PCR buffer (10µl/well), dNTPs (as an equimolar solution dATP, dCTP, dGTP and dTTP) (2µl per well) primers (0.2µl/well), enzyme mix (2µl per well) and water (to 50µl per well) was prepared in a clean PCR hood. 40µl of the master mix was then added to each well containing a cell. The PCR plate containing single sorted B cells, primers Table 2-4 (1 and 2) and reverse transcription mix was heated to 50°C for 30 minutes, and then cycled at 95°C (1 minute), 55°C (1 minute) and 72°C (1 minute) for 50 times each, followed by a final extension for 10 minutes at 72°C.
The RT-PCR reaction was then aliquoted into three wells, to allow reamplification of heavy and light chains, using specific nested primers (shown in Table 2-4 3 and 4). Annealing temperatures and cycling times were optimized using serial dilutions of mRNA from an EBV transformed B cell line (1:10-1:10000). A gradient PCR program was used to determine optimum primer annealing temperatures. The gradient temperature was set from 50-60°C. The optimum temperature range for all three primer mixes to anneal was 55-56°C.

Optimization of cycling temperatures revealed that both Heavy and Lambda chains could be amplified at the same cycling conditions and therefore in the same plate, using a 60°C annealing temperature, whilst Kappa chains required a lower temperature of 58°C, so was amplified independently. The minimum number of cycles required was 50 cycles in the RT-PCR reaction and a further 30 in the nested follow up PCR.

Figure 3-1: Scheme showing stages in amplification of immunoglobulin V region genes from single sorted IA-2 reactive human B cells.

Reactions were performed in the order shown in Figure 3-1. Three RT PCR reactions were run per cell; one for lambda light chain (λ), one for kappa light chain (κ) and one for gamma heavy chain (γ). The templates created by RT-PCR were transferred to three sets of wells in a 96-well plate. Each well contained 0.5µl of each 20µM 5’ nested primer, and 0.5µl of a 20µM 3’ constant region nested primer, 10µl GoTaq G2 buffer, 1µl dNTPs, and 1µl GoTaq G2 polymerase, with water to 50µl and 2µl single cell PCR product. The constant light chain primers, (CL2 and CK1Z) were taken from Burton and Barbas (Burton and Barbas 1994) and all are shown in Table 2-4. Products from the nested PCR reaction were electrophoresed, in a 2% TAE- Agarose gel containing ethidium bromide. Bands were visualized using a transilluminator. Bands were excised using a scalpel and gel purified by freezing for 5 minutes, followed by a centrifugation step, performed at 4000rpm, using the Freeze N’ Squeeze system (Bio Rad).
3.3.6 **Ligation and sequencing of PCR products:**

All appropriate bands were ligated into pGEM-T-easy (Promega, UK). Ligation was performed as detailed in section 2.3.2. Ligations were transformed into XL1B *E.coli* cells, and grown up overnight on ampicillin selective LB-Agar plates. Several colonies were picked into LB-Broth containing ampicillin, and grown at 37°C, overnight. Plasmids were isolated from overnight cultures using a Bio-Rad (USA) Quantum prep kit; DNA was measured at 260nm, using a Nanodrop device. The presence of inserts was verified by digestion with EcoRI, which cuts on either side of the insert, and gel electrophoresis. Samples were sent to Source Bioscience (Cambridge, UK) for sequencing using the SP6 promoter sequence, upstream of the insert.

The individual heavy and light chain sequences were compared to those in the VBASE2 database (www.vbase2.org) (Retter, Althaus et al. 2005); a database of germ-line variable genes from the immunoglobulin loci of both human and mouse origin. All variable gene sequences are extracted from the EMBL-Bank (Retter, Althaus et al. 2005). Sequences were aligned with germline genes presenting the greatest degree of homology. Sequence homology values were determined from the alignment of sequences going from the beginning of FR1 to the end of FR3; this allowed determination of functional IgG sequence.

3.3.7 **Preparation of antibody fragments:**

An important component in the creation of a functional scFv antibody fragment is the linker sequence. The linker sequence used in this case is the traditional (Gly3Ser)4 sequence, slightly shorter than that designed by Huston et al (Huston, Levinson et al. 1988). A slightly shorter linker favors scFv formation, over the dimeric diabody formation produced by shorter (8-12 amino acid) linker sequences (Holliger, Prospero et al. 1993). The linker sequence was incorporated into the primers.
Figure 3-2: Map of pFLAG vector. pFLAG CTS has the Omp-A periplasmic tag upstream of target sequence.

### Table 3-1: Primers for cloning into pFLAG CTS

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>B96scFvpFlagF</td>
<td>GAATTCCAGTCTGCCCTGACTCAGC</td>
</tr>
<tr>
<td>B96scFvpFLAGR</td>
<td>AGATCTTGAGGAGACGGTGACCAG</td>
</tr>
<tr>
<td>220VL2EcoRI</td>
<td>GAATTCGCCCTGAAGCTCCCTCCCTCGTG</td>
</tr>
<tr>
<td>220VH5BGL2</td>
<td>AGATCTTGAGGAGACGGTGACCAG</td>
</tr>
<tr>
<td>220VL8EcoRI</td>
<td>GAATTCAGACTGTTGGTGACCCAGGAGGCC</td>
</tr>
<tr>
<td>220VH3Bgl2</td>
<td>AGATCTTGAGGAGACGGTGACCAG</td>
</tr>
</tbody>
</table>

Heavy and light chain pairs were amplified using primers containing restriction sites for EcoRI and BglII restriction enzymes (shown in Table 3-1) added to permit digestion and ligation into an expression vector: pFLAG-CTS from Sigma (UK) (shown in Figure 3-2). Heavy and light chains were amplified separately, and then combined in an overlap reaction. Overlap products were gel purified, and ligated into pGEM-T-easy for sequencing. Plasmids containing scFvs of the correct sequence were digested with EcoRI and BglII, as was the pFLAG-CTS vector. Digested inserts and vectors were gel purified, and ligated overnight, prior to transformation into XL1B cells. Plasmids were prepared from overnight cultures, and digested to verify that scFv sequences were inserted. Plasmids with inserts were then transformed into BL21 cells for protein expression.
3.3.8 Protein Expression in pFLAG-CTS
Single colonies of transformed BL21 cells were picked into LB-Ampicillin, and grown up for four hours, in a shaking incubator (New Brunswick) at 37°C. A 200ml LB-Ampicillin culture was spiked with the overnight culture, and grown with shaking at 37°C until the culture reached an OD600 of 0.6, as an indicator that the cells have entered the log phase of replication. 200µl of 100mM IPTG was added to each culture, and protein expression was performed overnight at 20°C. Cultures were spun down at 10 000rpm, at 4°C.

3.3.9 Purification of scFvs:
Bacterial pellets were resuspended in PBS containing dithiothreitol (DTT), phenylmethanesulfonylfluoride (PMSF) and benzamidine. Cells were lysed by addition of 1mg/ml lysozyme, and mixed at 20°C for 30 minutes. Triton X-100 was added to precipitate DNA, lysates were mixed briefly, and then DNase1 added, and mixed for 10 minutes. Lysates were spun at 10000rpm, for 10 minutes, to remove cellular debris. Lysates were passed through a 0.4μM filter, and added to pre-washed anti-dykddddk tag LS affinity gel (BioLegend), an anti-FLAG tag antibody-coated resin, capable of binding and immobilizing FLAG-tagged fusion proteins. Lysates were rolled with gel for three hours, at 4°C, then unbound proteins were removed, and resin washed in ten bed volumes of PBS.

3.3.10 Testing Affinity of scFvs:
Gel bound scFvs were incubated with 20 000cpm of 35S-methionine labelled antigen: IA-2ic, GAD65 and ZnT8. 20μl of 50% gel/PBS slurry was added to 20μl 20 000cpm radiolabeled antigen and incubated overnight shaking at 4°C. Gel/Antigen mixtures were then transferred to blocked filter plates, and washed through with IMP buffer followed by H2O. Plates were dried at 37°C. 100μl of Microscint was added to each well, and bound radiolabel counted using a Chameleon scintillation counter (Hidex, UK).

To assess the effects of single amino acid substitutions on scFv binding of the IA-2 antigen, radioligand binding assays were also performed using translates incorporating single alanine substitutions of amino acids believed to contribute to the formation of the central PTP region epitope: 799, 835, 858, 836, 875, 879, 880, 954, 956 and 957. Substitutions were performed previously by myself, Diba Debnath, Dr. Kerry McLaughlin and Dr. Sarah Weenink.
### 3.4 Results:

#### 3.4.1 Patient selection:

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sample date</th>
<th>Ethnicity</th>
<th>Duration of diabetes</th>
<th>Sex</th>
<th>Age at onset</th>
<th>Yield of cells/ml (\times 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>12/2/2013</td>
<td>White British</td>
<td>Recent onset</td>
<td>Female</td>
<td>13</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 3-2: Categorisation of the patient sample used for B cell separation. 220 was a female recent onset patient, age 13, HLA type DR3/DQ2, recognising PTP and JM epitopes within the IA-2 molecule, with high affinity for GAD65 also.

Patient sample 220 was defined as a high affinity IA-2 positive sample, recognizing the central and JM regions of the molecule, and also GAD65, as shown in Table 3-2. This sample was selected for use in this experiment as it had high yields of lymphocytes, and had antibodies recognizing several different epitopes within the IA-2 molecule.

#### 3.4.2 FACS purification of IA-2-specific peripheral blood B cells from Type 1 diabetic patients

![Flow cytometric analysis of IA-2 positive IgG (CD27) positive B cells from an antibody negative patient sample and an antibody positive patient sample](image)

Figure 3-3: Flow cytometric analysis of IA-2 positive IgG (CD27) positive B cells from an antibody negative patient sample and an antibody positive patient sample (performed by Dr Kerry McLaughlin).

In order to isolate only IA-2 reactive B cells, Dr. McLaughlin used two consecutive sorts, the second at higher stringency in order to first isolate a large number of cells which recognized the IA-2 antigen, followed by a more specific sort, allowing purification of single IA-2 positive B cells. This process yielded 9 B cells labeled with the fluorescence-tagged IA-2, which were snap frozen on dry
ice. Not all cells seen in the FACS plot could be sorted, due to the limitations of flow rate; if two cells are too close together in the system, both will be binned. Whilst only a small proportion (0.01%) of cells labeled with anti-GST tag (recognizing bound IA-2) in the antibody positive patient, this proportion is significantly reduced in the antibody negative patient (0.003%), shown in Figure 3-3.

3.4.3 **Optimization of PCR using serial dilutions of mRNA extracted from B cell lines:**

![Image](image.png)

Figure 3-4: Optimisation of PCR annealing temperatures, using gradient PCR on diluted mRNA samples. Results demonstrate that a temperature of 54-56°C is appropriate for annealing of all primers, with low concentrations of mRNA.

In order to determine if it was possible to use RT-PCR to amplify IgG heavy, Kappa and Lambda sequence from low copy number templates, the RT-PCR reaction was optimized using mRNA extracted from the DS329 cell line (Chapter 2), diluted to 0.00375µg per well. Optimization was performed with a static concentration of primers, using a gradient for the annealing temperature of the primers, ranging from 54°C to 60°C. This optimization of PCR cycling demonstrated that the optimal temperature for annealing of all primer sets shown in Table 2-4 1-4, both kappa, lambda and heavy chains, was between 54-56°C, as can be seen in Figure 3-4. Using primer specific RT-PCR prevents the production of multiple copies of cDNA from mRNA within the cell, therefore the reaction must be optimized to maximize the possibility of generating transcripts from any possible primer pair in the mix.
3.4.4 Testing of conditions for RT-PCR amplification of immunoglobulin heavy and light chains

Following optimization of the annealing temperature on B cell mRNA, 23 wells of a plate of single IgG (CD27+/CD19+) B cells were amplified using mixed primers. Cells were subjected to one round of RT-PCR using primers from Table 2-4 (1 and 2), and a 2µl aliquot of the reaction was then subjected to a further 40 cycles of nested PCR. 10µl of the nested PCR reaction was electrophoresed, and visualized under a transilluminator, as seen in Figure 3-5. This first attempt at single cell PCR yielded one pair of heavy and light chains (lane 23) and one unpaired kappa chain (5).

![Figure 3-5: Initial PCR using single B cells not selected for IA-2. Reaction yielded one pair of heavy and lambda chains (Lane 23) and one unpaired kappa light chain (Lane 5).](image)

3.4.5 Sample 220: single selected IA-2 GST labeled B cell PCR

In order to generate IA-2 reactive monoclonal antibody fragments, the nine IA-2 GST-labeled B cells were isolated from total B cells of sample 220 and individually subjected to the RT-PCR protocol. A first round of PCR generated several faint bands at 200-250bp. These bands were excised and purified using a Bio-Rad Freeze N’ Squeeze kit, described in Chapter 2. Bands were then reamplified using the nested primer mix, for heavy, kappa or lambda as appropriate (Table 2-4, 3 and 4). Re-amplification produced one lambda light chain, from the VL3 family. The original RT-PCR reaction was then reamplified using a slightly lower annealing temperature of 59ºC. This gave several heavy chain bands shown in Figure 3-6. All bands were purified and ligated into pGEM-T-easy. Four clones gave validated heavy chain sequences: H1, H2, H3, H4 and H9.
No bands were seen for the first kappa reaction (data not shown) and not all cells yielded a V lambda band. Therefore an aliquot of the PCR reaction was taken for a subsequent reamplification, seen in Figure 3-7. Subsequent analysis of light chains yielded two more light chain products, both of V lambda class which ran at 220bp and were further verified by sequencing. Reamplified Kappa chains did not produce any valid IgG sequence.
Table 3-3, demonstrated that two heavy chains had light chain partners. H1 has no productive match (L1 amplified a nonspecific product). H3 matches L3, and H4 matches L4, giving two sets of matching sequences, H3 L3 (VH3 family, VL8 family) and H4 L4 (VH5, VL2). The lambda 3 amplification gave two light chain sequences, 3A (VL8) and 3 B (nonproductive VL7).
Table 3-3: DNA and predicted protein sequences generated from IA-2 reactive B cells isolated from sample 220.

<table>
<thead>
<tr>
<th>B cell Number</th>
<th>DNA/ predicted protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Heavy chain, 220 1 VH1)</td>
<td>DNA: GAGGTGCAGCTGGGCTGAGTGAAGGCTGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTTTCAGCTGGGTGCGACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAGAAAACTACGCACAGATGTTCCAGGGCAGATCAGTACGGCAGAATCCACGACAGCAGCCTACATGGAGCTGAGCAGCC TGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGAGGATATGGTTCGGGGATTTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG</td>
</tr>
<tr>
<td>2 (Heavy chain, 220 2 VH3)</td>
<td>DNA: GAGGTGCAGCTGGGCTGAGTGAAGGCTGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTTTCAGCTGGGTGCGACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAGAAAACTACGCACAGATGTTCCAGGGCAGATCAGTACGGCAGAATCCACGACAGCAGCCTACATGGAGCTGAGCAGCC TGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGAGGATATGGTTCGGGGATTTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG</td>
</tr>
</tbody>
</table>

133
<table>
<thead>
<tr>
<th>Region</th>
<th>DNA sequence</th>
<th>Protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (Heavy chain, 220 3 H3)</td>
<td>AGAGTGCAAGCTGGTGAGTCTGGGGGAGCGTGCTGCAGCCCTGGAGGCTCCTGAAGCTCT CTTTGCAAGCTGGTGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAAGCTCTTGTGCAGCCTCTGGATTCACCTTCAGTAGGTTTGGCATGCACTGGGTCCGCCACGCTCCAGGCAAGGGGCTGGAGTCCGTGGCAGCTATATCATATGATGGAGGTAGTCAATACTATCCAGAC TCCGCTGAAGGGGCGCCTTCACCATCTCCAGACACAGACAATCCAAGAACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGCACAAAACCAACTCAAGAGTCTTATGAACCCCCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCAG</td>
<td>RVQLVESGGGVVQPGRSLKLSCAASGFTFSRFGMHWVRHPGKLESVAISYDGGSQYYPDSSVKGRFTISRDNSKNTLYQMDLSRLAEDTAVYYCTKPTQESYEPFPDIWGQGTMVTSS</td>
</tr>
<tr>
<td>3 (Light chain, 220 3 VL8)</td>
<td>CAGACTGTGGTGACCCAGGAGCCATCGTTCTCACAGTGCTCCCTGGAGGAGGACACGTGCACACTCAGTCTGGGCTTAAACTCTGGCTCAGTCTGACTAGTTACACACCAAACACTGCCTCTCTGGGCTCCTGTACGGCTTCTGTGGGCTCCATCCTTGGGAACAAAGCTGCCCTCACCATCACGGGGGCCCAGGCAGATGATGAACTGATTATTACTGTGTGCTGTATATGGGTAGTGGCATTTCAGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAG</td>
<td>QTVVTQEPSFSVSPGGTVTLCGLNSGSVSTSYPSWYQQTPQAPRTLIYSTNTRSSGPDRFSGSILGNKAALTITGAQADDESYYCVLYMGSGISVFGGTTKLTVL</td>
</tr>
<tr>
<td>4 (Heavy chain, 220 4 VH5)</td>
<td>GAGGTGCAGCTGGTGAGTCTGGGGGAGGCGTGCTGCAGCCCTGGAGGCTCCTGAAGCTCTCCTGAAGGGTTCTGGATACAGCTCTACCTA</td>
<td>EVQLVQSGAEVKPGESLTISSGYSFTTYWIDWVRQMHPGKLEWMGKIDPSDSYNTNYPSEQGHVTISGDKSTAYLQWTSKASDTAMMYCARRNPFGHDAFDIQWGQGTMVTSS</td>
</tr>
<tr>
<td>4 (Light chain, 220 4 VL2)</td>
<td>DNA: GCCCTGACTCAGCCTCCTCCGTGCCGGTCTGGACAGTCAGTCACCATCTCCTGCACTGGAACCAGCCGTGACGTTGTCATTATGACAACCTGCTGGTACCAGCAGCCCCCAGGCGGCCCCAAAACCTCTGATTTTTGAGGTCAATAATCGGCCCTCAGGGGTCCCTGATCGCTTCTCTGGGTCCAAGTCTGCAACAGCCTCCCTCGACCATCTCTGGGCTCCAGGCTGAGGACGGCTGATTATTACTGCTGCTCATTTACAAGCAGCAACACTTTTGATTCGGCGGAGGGACCAAGTGACCGTCCT</td>
<td></td>
</tr>
<tr>
<td>4 (Light chain, 220 4 VL2)</td>
<td>Protein: ALTQPPSVSGPQSVTISCTGTGSDVGHYDNRSWYQPPGTAPKLLIFEVNNRPSGVPDRFSGSKSANTASLTISGLQAEADYYCCSFSSNTLVFGGGLTVL</td>
<td></td>
</tr>
</tbody>
</table>
### 3.4.6 Germline Alignments Generated:

<table>
<thead>
<tr>
<th>B cell Number</th>
<th>Sequence name:</th>
<th>Germline matches</th>
<th>Junction Matches</th>
<th>Diversity Matches</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Heavy chain)</td>
<td>220 H1 (IgG VH1)</td>
<td>humlGHV034, humlGHV237, humlGHV035, humlGHV020, humlGHV182</td>
<td>X86355 IGHJ4<em>02, X86355 IGHJ3</em>02, X86355 IGHJ5*02</td>
<td>X13972 IGHD3-10<em>01, X93615 IGHD3-10</em>02, J00233 IGHD2-8*02</td>
</tr>
<tr>
<td>2 (Heavy chain)</td>
<td>220 H2 (IgG VH3)</td>
<td>humlGHV187, humlGHV157, humlGHV290, humlGHV045, humlGHV313</td>
<td>J00256 IGHJ2<em>01, J00256 IGHJ1</em>01, M25625 IGHJ4*03</td>
<td>X93618 IGHD3-3<em>02inv, X13972 IGHD3-10</em>01inv, X13972 IGHD3-3*01inv</td>
</tr>
<tr>
<td>3 (Heavy chain)</td>
<td>220 H3 (IgG VH3)</td>
<td>humlGHV195, humlGHV047, humlGHV199, humlGHV050, humlGHV029</td>
<td>X86355 IGHJ3<em>02, J00256 IGHJ3</em>01, X86355 IGHJ6*02</td>
<td>X93616 IGHD3-22<em>01, X13972 IGHD3-10</em>01, X93615 IGHD3-10*02</td>
</tr>
<tr>
<td>3 (Light chain)</td>
<td>220 L3A (IgG VL8)</td>
<td>humlGLV044, humlGLV010, humlGLV149, humlGLV011, humlGLV029</td>
<td>D87023 IGLJ3<em>02, M15642 IGLJ3</em>01, M15641 IGLJ2*01</td>
<td>No Diversity match</td>
</tr>
<tr>
<td>3 (Light chain)</td>
<td>220 L3B (IgG VL7)</td>
<td>humlGLV134, humlGLV167, humlGLV060, humlGLV064, humlGLV070</td>
<td>D87023 IGLJ3<em>02, M15642 IGLJ3</em>01, M15641 IGLJ2*01</td>
<td>No Diversity match</td>
</tr>
</tbody>
</table>
Amplification of single B cells from sample 220 produced two possible functional pairs of heavy and light chain sequences (H4/L4, H3/L3A). In addition, two orphan heavy chains were generated in this study (H1, H2), and one out of frame rearrangement of a lambda chain, one of two alternative light chains, as it originated from well number 3.

### 3.4.7 Assembly of heavy and light chain pairs amplified from single B cells as an scFv

To test antigen binding of recombinant antibody fragments generated by RT-PCR of single B cells, light and heavy chains must be combined to make a functional antibody fragment. Primers were designed for amplification of V regions with restriction sites to allow assembly of an scFv fragment joined by a linker. Constructs were cloned with a FLAG tag in the pFLAG-CTS vector. Amplified products, using primers shown in Table 3-4, are shown in Figure 3-8, which were then linked using an overlap PCR reaction.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>220VL2Mlu</td>
<td>AACCGCTATGCTGCCCTGACTCAGCCTCCCTCCGT</td>
</tr>
<tr>
<td>220VL2GSrev</td>
<td>CGACCCACACGCCGAGCCACGCCAGGACGTCAGCTTG</td>
</tr>
<tr>
<td>220VH5GSFor</td>
<td>TCGGCCCAGTTGCTGGTGTCGGTGGGAGGATCGAGCTTGAAGAGACGGTTACCATTGT</td>
</tr>
<tr>
<td>220VH5HindIII</td>
<td>TCAAGCCTTCTGTGCTCATCGCTGCTTTGTAGTCTGAAGAGACGGTTACCATTGT</td>
</tr>
<tr>
<td>220VL8Mlu</td>
<td>AACCGCTATGCTCAGACTGTGGTGGACCCAGGAGCC</td>
</tr>
<tr>
<td>220VL8GSREV</td>
<td>CGACCCACACGCCGAGCCACGCCAGGACGTCAGCTTG</td>
</tr>
<tr>
<td>220VH3GSfor</td>
<td>TCGGCCCAGTTGCTGGTGTCGGTGGGAGGATCGAGCTTGAAGAGACGGTTACCATTGT</td>
</tr>
<tr>
<td>220VH3HindIII</td>
<td>TCAAGCCTTCTGTGCTCATCGCTGCTTTGTAGTCTGAAGAGACGGTTACCATTGT</td>
</tr>
</tbody>
</table>

Table 3-4: Primers designed for scFv overlap PCR, with MluI and HindIII restriction sites (Bold), and GS linker overlap sequence (underlined).
Figure 3-8: Reamplification of heavy and light chains, using specific primers, shows B cell 3 VH3, and partner light chain VL8, B cell 4 VH5 with partner light chain VL2 and a monoclonal antibody to GAD65, B96, heavy chain and light chain. Also shown are heavy and light chain amplifications for DS329, VH3 VL2. Restriction sites create a slightly larger product which runs at 250bp.

The FLAG tag allows direct binding of the antibody tag, permitting direct binding of antigen to an anti-FLAG antibody coated resin, which is capable of binding a C-terminal FLAG tag, leaving the antibody fragment free to bind target antigen. scFv fragments were generated and ligated into pGEM-T-easy for subcloning into pFLAG-CTS. It was necessary to reamplify entire assembled scFv products for ligation into pFLAG-CTS. Specific primers, incorporating restriction sites BglII and EcoRI, were used to amplify scFvs. Reamplified sequences were then ligated into pGEM-T-easy, digested, and ligated into an IPTG induction vector with a C-terminal FLAG tag, pFLAG-CTS. The FLAG tag is an epitope tag consisting of eight amino acids Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK). This tag is small enough not to interfere with antibody fragment binding, and binds specifically to FLAG resin.

After amplification of separate single heavy and light chains, PCR products were combined at an equivalent molar concentration. An overlap PCR reaction using the forward primer for the light chain and the reverse primer for the heavy chain generated a product of the appropriate size, as shown in Figure 3-9.

Figure 3-9: Overlap PCR to generate scFv fragments for 220 VH5(cell 4, VH5VL2), 220 VH3 (cell 3, VH3VL8), DS329 (VH3VL2) and B96 (VH3VL2). Two reactions were performed for each overlap PCR, one using purified PCR products, and the other using product taken directly from the PCR mix. The reaction often generated two bands, one at 750bp, representing the assembled scFv and a lower band, just below 500bp, which represents reamplified single heavy and light chains.
3.4.8 Sequencing of scFvs:

Products successfully amplified with the linker overlap were ligated into pGEM-T-easy for sequencing. Two scFv sequences from the sample of patient 220 were confirmed by sequencing. Both DNA and predicted protein sequences are shown below in Table 3-5. These sequences had appropriate restriction sites for cloning into the pFLAG CTS vector.

Table 3-5: DNA and predicted protein sequences of 220 scFv fragments.

<table>
<thead>
<tr>
<th>220 VHS (cell 4) VL2VHS scFv DNA sequence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAATTCGCCCTGACTCAGCCTCCCTCCGGTGTCCGGGTCTCCTGGGCAAGCTGACCGGTGGCCTGGGTGGCGGCGGATCGGAGGTGCAGCTGGTGCAGTCGGAGCAGAGGTGAAAAAGCCCGGGGAGTCGCTACAATCTCCTCTGTTCTGATACGCTTCAGGTTCTGGATACAGCTTCACCACCTACTGGATCGATTGGGTGCGCCAGATGC</td>
</tr>
<tr>
<td>Protein:</td>
</tr>
<tr>
<td>EFALTQPPSVSGSPGQSVTISCTGTGSRDVHYDNRWSWYQQPPGTAPKLLIFEVNNRP</td>
</tr>
</tbody>
</table>
In order to confirm that the isolated antibody sequence has specificity for the IA-2 antigen, and to validate the antigen specific sorting method, the scFvs generated in this study were digested and ligated into the bacterial expression system previously used for antibody fragments generated from monoclonal antibody cell lines. Once ligated into the pFLAG-CTS vector, scFv expression was induced by the addition of IPTG. Expression was performed overnight, cells were pelleted and lysed, and scFvs were purified using Anti-DYKDDDDK Tag (L5) Affinity Gel (Bio Legend).

Verification that the expressed protein products were of appropriate size was achieved by polyacrylamide gel electrophoresis, followed separately by Coomassie staining, and by anti-FLAG tag Western blotting (Figure 3-10). Samples of the bacterial cell lysate, the flow through fraction from the affinity column and the eluted protein were reduced using DTT and heating, and run in a 12% Bis-Tris (Invitrogen) gel, in MES running buffer (Invitrogen). One gel was then subjected to a transfer to PVDF membrane (GE Life Sciences), whilst the other was stained with Coomassie R-250. The flow through fraction was not completely cleared of FLAG tag labeled protein, suggesting that more resin should be used in future purification protocols.
Antigen specificity for IA-2 was determined using a competition radioligand binding assay. The scFvs were dialyzed into PBS and concentrated to 250µg/ml. 5µl of concentrated scFv or PBS was added to V005 serum (healthy control) and 220 serum (serum from the patient the fragments were generated from). Concentrated 96/3 scFv was also used as a control. As shown in Figure 3-11, both scFv generated from B cells isolated from PBMCs from patient 220 were capable of partially inhibiting serum antibody binding of IA-2.

Figure 3-11: Showing partial inhibition of 220 serum antibody binding of radiolabeled IA-2 upon addition of 220 3 VH3 or 220 4 VH5 scFv. No inhibition is seen upon addition of 96/3 scFv. Also showing lack of binding of negative control serum V005.
As a further test of inhibition, scFvs were used to inhibit binding of three IA-2 specific monoclonal antibodies, and one rabbit polyclonal. As shown in Figure 3-12, all three scFvs were capable of inhibiting binding of both 96/3 and M13 monoclonal antibodies. Partial inhibition was seen for DS329, and R2B2, a rabbit polyclonal antibody recognizing predominantly the JM domain of IA-2, was used as a negative control.

![Graph showing inhibition of monoclonal antibody binding by addition of IA-2 reactive antibody fragments generated from sample 220. Both 200 scFv fragments are capable of significantly inhibiting binding of IA-2 translate by monoclonal antibodies. The 96/3 scFv is included as a positive control for inhibition.](image)

**Figure 3-12:** inhibition of monoclonal antibody binding by addition of IA-2 reactive antibody fragments generated from sample 220. Both 200 scFv fragments are capable of significantly inhibiting binding of IA-2 translate by monoclonal antibodies. The 96/3 scFv is included as a positive control for inhibition.

To verify specific binding of IA-2 antigen, the scFvs were tested against three major islet autoantigens, IA-2, GAD65 and Znt8, using a direct binding assay: scFvs bound to anti-FLAG resin were incubated with radiolabeled antigen overnight, transferred to a filter plate, washed and counted the next day.

Testing showed that both scFvs generated from isolated B cells were capable of binding IA-2 directly, when bound to anti-FLAG resin. None of the scFvs were capable of binding an irrelevant antigen, ZnT8, or GAD65, as seen in Figure 3-13.
Further analysis of binding, using truncated transcripts to represent specific epitope regions of the IA-2 molecule, shown in Figure 3-14, demonstrated that both monoclonal antibodies generated from IA-2 specific B cells isolated from patient sample 220 showed similar binding patterns: both bound efficiently to IA-2 IC (whole molecule) and to IA-2 PTP (a fragment representing the protein tyrosine phosphatase region of the molecule). Neither antibody bound a chimeric construct representing the JM region, although one showed very weak binding to the central region only fragment, CR. These results demonstrate that both antibodies recognize a central region epitope, seen in polyclonal patient sera, and other human monoclonal antibodies.
Figure 3-14: Binding of 220 scFvs to truncated IA-2 transcripts: Both 220 scFvs bind efficiently to the PTP domain of IA-2, but binding is reduced with a transcript missing the C-terminus of the molecule. Neither scFv binds the JM domain.

3.4.9 Epitope Mapping of 220 scFv:
A number of mutated transcripts of the PTP domain of IA-2, each with a single amino acid substitution in the putative epitope region, had been generated for earlier studies. These transcripts were used to identify amino acids that may contribute to the formation of epitopes recognized by the scFv, and thereby determine key amino acids for binding of human sera. Among the amino acid substitutions tested were those previously implicated in 96/3 antibody binding (Glu836, Trp799 and Asn858) one amino acid (Tyr835) thought to lie adjacent to Glu836 within IA-2 PTP domain, and a further six amino acids within a region of the molecule suggested to contribute to the formation of a second epitope within the PTP domain (D. Debnath, unpublished) Trp875, Gly879, Thr880, Arg954, Lys956 and Asp957. Analysis of antibody binding of the monoclonal antibodies to mutant IA-2 constructs showed that substitution of Glu836 strongly inhibited binding both scFvs, as with other central region reactive antibodies (Weenink, Lo et al. 2009).
Figure 3-15: Showing inhibition by substitution of single amino acids in the PTP domain of IA-2. All three scFvs are inhibited by substitution of amino acid 836, however both 220 scFvs are affected by substitution of amino acid 957, and the VHS scFv is also inhibited by substitution of amino acid 799.
As shown in Figure 3-15, substitution of amino acid 836 inhibits binding of all three scFvs. This amino acid has previously been shown to affect binding of serum antibodies (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009). Both 220 scFvs were also inhibited by substitution of the 957 amino acid, suggesting a requirement for the C-terminus of the IA-2 molecule. 220 4 VH5 also show some inhibition upon substitution of the Trp799 amino acid, which has no effect on both other scFvs.

3.4.10 Comparison with epitopes defined in previous studies:

Two studies have previously been published, identifying key amino acids recognized by human monoclonal antibodies to IA-2 (Weenink, Lo et al. 2009; Catani, Walther et al. 2015; McLaughlin, Richardson et al. 2015). Inhibition of scFv binding by amino acid substitutions performed in this study was compared with data obtained in these two previous studies, to generate a clearer picture of epitopes involved in central region binding. Comparison of data from all four studies demonstrated the importance to the glutamate 836 residue, which inhibited binding of all six monoclonal antibodies (200VH3, 220VH5, 96/3, DS329, M13, A6 and C11). Not all antibodies were inhibited by substitution of trp799, and only the 220 scFvs can be seen to be inhibited by substitution of amino acid 957.

3.5 Discussion

As demonstrated by Reichert monoclonal antibodies are used for research, diagnosis of disease, and therapy; these molecules are amongst the most rapidly expanding numbers under production in recent years (Nelson and Reichert 2009). There is a wide array of possible formats for these molecules, and expression systems used (Frenzel, Hust et al. 2013). Generation of functional antibody fragments using a bacterial expression system is dependent on both length and composition of linkers, inclusion of a periplasmic tag, and expression method. Kortt et al (Kortt, Lah et al. 1997) demonstrated that the formation of a functional antigen binding site between variable domains requires a linker that can span at least 32–34 Å (Huston, Levinson et al. 1988; Knappik and Pluckthun 1995; Kreberger, Bornhauser et al. 1997; Volkel, Korn et al. 2001), which suggests a requirement of 13 -15 amino acids in the linker sequence.

The aim of this study was to produce new functional monoclonal antibody fragments which could be expressed at high concentrations for use in characterization of epitopes recognized in type 1 diabetes. Development and validation of a method for selecting individual B cells capable of recognizing the IA-2 antigen holds promise for future studies. Using antigen specific sorting, for IA-2 positive B cells gave a greater chance of generating IA-2 reactive monoclonal antibody fragments than selecting B cells from the total B cell pool obtained from patient samples. Unfortunately, this method does limit the number of cells recovered after sorting. As shown by Catani et al (Catani,
Walther et al. 2015), detection of autoreactive B cells using non pre-screened cells is time consuming; using single cells pre-selected for antigen reactivity greatly improves the chances of success. The study produced two antibody fragments, from only 9 cells isolated by FACS, capable of binding the IA-2 antigen.

Single IA-2 positive B cells are present at extremely low frequencies in the periphery: isolation of these cells requires binding of a labelled antigen with minimal background. This approach allows detection and isolation of rare B cells from the periphery. This study draws on techniques used by the Wucherpfennig group (Franz, May et al. 2011), studying antibodies to tetanus toxoid. This group used streptavidin labelled tetramers, which were not optimal for detection of auto reactive B cells in this study, as shown by Dr. Kerry McLaughlin (personal communication).

The generation of non-functional sequences and orphan chains is not surprising, bearing in mind that orphan chains may arise because the PCR is not 100% efficient. The conditions used for amplification of kappa light chains may not have been optimal because of the lack of products with these primers. In one cell, sequences for both productive and nonproductive VDJ rearrangements were obtained. One out of the four B cells which gave sequence contained both functional and non-functional rearrangement products. Lanzavecchia et al (Giachino, Padovan et al. 1995) demonstrated the presence of dual immunoglobulin presenting B cells in the peripheral repertoire of mice and humans. This observation has recently been confirmed by De Kosky et al (DeKosky, Kojima et al. 2014), and their research suggests that whilst only a small number of cells express two forms of IgG (0.4%), and some do contain mutated, nonfunctional transcript.

The ability of scFvs generated in this study to bind radiolabeled antigen suggests that the design of the linker and the bacterial expression system used were suitable, in this instance for production of autoantibody fragments. Lack of binding of irrelevant antigen implies the products are antigen specific, validating the approach for selection and purification of IA-2 specific B cells from patients’ blood.

Previous studies of autoreactivity to the IA-2 antigen have identified two major regions of autoantibody reactivity; the juxtamembrane and PTP domains of IA-2. Within these regions there may be several defined epitope regions. This study reinforces the concept that amino acids in the region close to amino acid 836 of IA-2 are extremely important for IA-2 autoantibody binding in type 1 diabetes (Weenink, Lo et al. 2009). Study of the epitope recognition pattern of both scFvs generated in this study suggests that they also recognize a central region epitope centered on the
E836 amino acid, however, both 220 scFvs also require the presence of the c-terminus of the IA-2 molecule to bind, and may recognize a wider conformational epitope than that recognized by 96/3.

Successful generation of two monoclonal antibody fragments from single B cells demonstrated the flexibility of the system devised for cloning and expression of thee fragments.
Chapter 4  Generation of Variable Heavy (VH) chain transcripts from archival tissue

4.1 Introduction:
To date, studies on autoantibodies in Type 1 diabetic patients have been restricted to those in peripheral blood. The specificity of these autoantibodies can be determined, and their targets are located within the pancreatic β-cell. The ability to define autoimmune responses local to the pancreas is severely limited due to the rarity of tissue available for study. There are nevertheless collections of archival post mortem tissue, fixed and embedded in paraffin wax, which have been used almost exclusively for immunohistochemical studies of the infiltrate in the diabetic islet. Recent advances such as laser capture microdissection has allowed the dissection of islets from frozen pancreas sections (Marselli, Sgroi et al. 2009; Sturm, Marselli et al. 2013). Dissection of single fixed islets from archival pancreas sections could permit profiling of cells present in the islet; or in the case of type 1 diabetes, profiling immune cells, in the islets or draining pancreatic lymph nodes. Some nucleic acids, including RNA, may be preserved by fixation and embedding of these tissue samples. Recovery of RNA from infiltrating immune cells within the pancreas would permit a more localized analysis of the immune response in diseased tissue of the human type 1 diabetic pancreas.

Previous results from immunohistochemical profiling of islet infiltrates (Willcox, Richardson et al. 2009) demonstrated high levels of CD8+ T cells in insulitic lesions. Lower levels of CD68+ macrophages were found; the group also found CD4+ T cells in infiltrates, but revealed that these cells were less common than either cytotoxic T cell or macrophages. CD20+ B cells were found in islets with higher levels of infiltration, but there were very few CD138+ plasma cells. The presence of B cells within the inflammation opens up the possibility of investigating the specificity of immune responses within the inflamed islet by cloning and characterizing antibody transcripts.

The increased sensitivity of RT-PCR technology has increased the interest in characterizing gene expression in archival formalin-fixed, paraffin embedded (FFPE) samples. Obtaining nucleic acids from archival samples can pose many problems, not least in that fixatives were not specifically designed to preserve them. However, several recent studies have extracted both DNA and RNA preserved in formalin and ethanol and paraffin embedded (Lewis, Maughan et al. 2001).

New methods for extraction of mRNA from FFPE tissues permit assessment of the quality of RNA preservation in tissue samples. Microdissection of islets from these tissue samples allows specific amplification of desired products, from identified groups of cells. Molecular analysis of FFPE samples
has to date been used to quantify gene expression in normal and abnormal tissues. This type of analysis can now be used for the analysis of RNA expression in particular groups of cells.

A recent study of genomic DNA extraction from umbilical cord sections gave good quality gDNA from samples stored for up to 19 years (Santos, Saito et al. 2008). The results of this study showed that good quality material depends on several variables, particularly fixative (Bouin’s or formaldehyde) and the method of dewaxing tissues, as use of xylene is thought to inhibit the action of Proteinase K, used to break down crosslinks in nucleic acids formed by fixation (Coura, Prolla et al. 2005). A later study (von Ahlfen, Missel et al. 2007) showed clear effects of processing on yields of RNA from archival samples:

1. Time to fixation: the amount of time a sample is left for prior to incubation with fixative. Differences in preservation of RNA between samples is more likely to be an indicator of tissue autolysis and processing prior to fixation rather than conditions of preservation post fixation.
2. Storage time of paraffin blocks/sections.
3. Conditions of storage of paraffin blocks.
4. The method used for isolation of RNA; RNA from whole sections was not adversely affected by incubation with proteinase K, but this period of incubation seemed sufficient to almost abolish recovery of amplifiable RNA from isolated islets.
5. Formaldehyde fixation inactivates endogenous tissue RNases: subsequent exposure to water of dewaxed sections may allow degradation of RNA by exogenous RNases.

Extraction of message (mRNA) from FFPE tissues has recently become, if not routine, then practicable. FFPE samples of breast cancer tissue have been analyzed to generate transcripts from RNA (Ma, Ambannavar et al. 2014). Krafft et al (Krafft, Duncan et al. 1997) demonstrated the feasibility of extraction of RNA from archival tissue, using either proteinase K or guanidine thiocyanate. This group achieved a 96% success rate in extracting RNA that was suitable for use as a template to amplify products as long as 197bp. A later study performed by the Korbler group (Korbler, Grskovic et al. 2003) optimized methods for obtaining good quality RNA from formalin fixed tissue, using xylene to remove paraffin wax, ethanol to remove solvent and an overnight digestion in Proteinase K. Both groups used standard end point PCR rather than real time PCR to generate products. Both duration of fixation and processing of tissues influence the success of amplifications performed. More recently, the Gerling group have profiled gene expression levels in type 1 diabetic
and non-diabetic samples from the nPOD databank: these samples were previously cryopreserved, allowing better preservation of RNA: the group extracted RNA from 40-60 islets per sample, and changes in levels of expression of genes associated with oxidative stress in diabetic islets, HLA class I, HNF4A regulated genes, genes targeted by EIF2 and mTOR pathways and genes involved in mitochondrial dysfunction (Kakoola 2014). Results of these studies suggest that some nucleic acids may be preserved, even in older samples.

Owens et al (Owens, Burgoon et al. 2001) demonstrated clear differences between the IgG heavy chains repertoire expressed at the site of inflammation in another disease, multiple sclerosis, and heavy chains isolated from peripheral blood. Heavy chain sequences isolated from the CNS plaque and periplaque white matter showed multiple somatically mutated clones of the same parental heavy chains. These chains were not found in peripheral blood, suggesting that they were the result of a CNS restricted, compartmentalized immune response, rather than B cells entering the brain from the blood. Sequencing of heavy chains from both the peripheral lymph nodes around the pancreas and islets would allow some degree of lineage tracing of B cell clones.

As shown in chapters 2 and 3, this study has previously amplified heavy and light chain sequences from mRNA from cell lines, and single sorted B cells. Using a similar technique, this section of the study attempts to manually micro dissect islets from both normal and recent onset type 1 diabetic pancreas. Preliminary work used actin and insulin genes to verify the presence of mRNA in the tissue dissected. The next step was to attempt to amplify transcripts representing immunoglobulin variable regions from both pancreatic peripheral lymph nodes and islets.

**4.2 Justification and Aims:**

Defining the specificity of autoimmune responses within the Type 1 diabetic pancreas is crucial for an understanding of disease pathogenesis and the development of therapies to prevent disease. Our knowledge of immune responses in human Type 1 diabetes is derived almost exclusively from studies in the periphery, owing to difficulties in accessing the pancreas in patients with active disease. Studies of the pancreas have been largely restricted to immunohistochemical observations on archival paraffin-embedded tissue. Should nucleic acids, including RNA, be preserved by tissue fixation and embedding, this may permit detailed analysis of immune responses in the diabetic pancreas through cloning of B cell and T cell receptors from the tissue.

The aim of this study was to assess the possibility of extracting and amplifying RNA from archival FFPE tissue samples from control and type 1 diabetic samples: this permitted the evaluation of the
potential for cloning immunoglobulin V-regions from B cells within islets and lymph nodes in sections of archival diabetic pancreas.

4.2.1 Methods:

4.2.1.1 Samples:
Samples were obtained from archival tissue through collaborations with groups in Glasgow, Exeter and Oxford. One recent onset type 1 diabetic patient and two control samples were obtained from Oxford (shown in Table 4-1). All three samples had been stored for 34-35 years. Tissue from the diabetic patient (OX039) was available fixed in both Bouin’s fixative and formal saline, although control sections were either fixed in Bouin’s (OX033) or formal saline (OX080). A formalin-fixed pancreas sample, A56, was also obtained from an organ donor from King’s College Hospital in 1998 with ethical approval for research into pancreas function and morphology from the King’s College Hospital Research Ethics Committee. Serial sections were cut at 5µm thick for immunohistochemistry and 10µm thick for amplification of transcripts by RT-PCR.

This allowed visualization of inflamed islets (if any), and staining for insulin and CD20. Availability of serial sections also permitted localization of islets and lymph nodes on serial thick sections.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sex</th>
<th>Age at death</th>
<th>Sample date</th>
<th>Date of disease onset</th>
<th>Duration of disease</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX039</td>
<td>Male</td>
<td>4 years</td>
<td>June 1980</td>
<td>June 1980</td>
<td>3 weeks</td>
<td>Diabetic ketoacidosis</td>
</tr>
<tr>
<td>OX033</td>
<td>Male</td>
<td>22 years</td>
<td>May 1979</td>
<td>n/a</td>
<td>n/a</td>
<td>Paragliding accident</td>
</tr>
<tr>
<td>OX080</td>
<td>Female</td>
<td>27 years</td>
<td>March 1980</td>
<td>n/a</td>
<td>n/a</td>
<td>Nephrectomy</td>
</tr>
<tr>
<td>E581</td>
<td>Female</td>
<td>10 years</td>
<td>n/a</td>
<td>n/a</td>
<td>new</td>
<td>n/a</td>
</tr>
<tr>
<td>Sc115</td>
<td>Female</td>
<td>16 months</td>
<td>n/a</td>
<td>n/a</td>
<td>3 days</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 4-1: Characteristics of patients used in this study.

Samples from Exeter came from a collection assembled by Dr. Alan Foulis in Glasgow. Two samples were used in this study, both with high levels of islet B cell infiltration, and visible pancreatic lymph nodes (PLNs).
4.2.1.2 Immunohistochemistry

FFPE tissue was available in 5µm thick sections, on untreated glass slides, from a recent onset type 1 diabetic patient provided by Dr. Anne Clark in Oxford. Control sections were cut onto polylysine coated slides. All samples were melted onto glass slides, dewaxed in xylene, and rehydrated in serial dilutions of ethanol. Endogenous peroxidases were blocked with H₂O₂, and nonspecific binding blocked with normal rabbit serum. Sections were ringed with wax using an ImmEdge wax pen (Vector Laboratories, Peterborough, UK), and incubated overnight at 4°C with primary antibody (rabbit anti CD20, DAKO at 1:1000). Antibody binding was detected using a DAKO Envision HRP/DAB rabbit/mouse kit (DAKO, UK). Sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Sections were visualized under a Nikon microscope.

4.2.1.3 Extraction of RNA from paraffin embedded sections

To evaluate the feasibility of RNA extraction from archival tissues RNA, was extracted from fixed tissue, cut directly into tubes for extraction using the Promega Reliaprep FFPE RNA extraction kit. 20µm sections were cut into 1.5ml Eppendorf tubes, and dewaxed using mineral oil. Cells were lysed overnight by heating to 56°C, then 10 minutes at 80°C. Samples were spun at 10 000rpm, and a 10µl aliquot taken for amplification and genomic DNA digested using DNase 1. RNA was then bound to a column, washed in four column volumes of wash buffer containing ethanol, and eluted in water. RNA yields were measured using a Nanodrop device (Thermo Scientific, UK).

Extracted RNA was subsequently amplified using a Qiagen Onestep RT-PCR kit, using mixed primers to amplify short (<120bp) products from actin and insulin. PCR products were further amplified using single primer pairs, with Go Taq G2 DNA polymerase, to determine levels of preservation of RNA in archival tissues. Products were analyzed on a 1.5% TAE agarose gel.

4.2.1.4 Manual microdissection of islets

Following successful amplification of RNA from whole sections, the feasibility of amplifying RNA from discrete areas of sections was tested. To manually dissect islets and PLNs, a protocol modified from the Morgan group in Exeter was used: sections were cut onto plain glass slides, at 10µm if possible (type 1 diabetic sections were pre-cut at 5µm). Sections were counterstained in hematoxylin (Mayer’s, Sigma, UK) and visualized under a x4 dissection microscope. Sections were kept hydrated with RNase free H₂O, and a 1¼ inch long, 21 gauge disposable syringe needle used to scrape away tissue from around the islet or PLN. Remaining tissue was then scraped into a 0.5ml Eppendorf tube containing 10µl of Qiagen buffer EB (10mM Tris-Cl, pH 8.5) using a fresh needle. Samples were
frozen at -20°C, if necessary, and extracted by heating to 56°C overnight, then 10 minutes at 80°C. Samples were spun at 10 000rpm, and a 10µl aliquot taken for amplification. Extracted samples were then subjected to PCR amplification. A trial of extraction using proteinase K (10U/islet) overnight at 56°C showed no benefit from Proteinase K treatment.

Samples obtained from Exeter were dissected on site by Dr. Pia Leete, and transported on dry ice in Qiagen buffer EB prior to storage at -80°C on arrival at King’s College London.

4.2.1.5 PCR amplification of actin, insulin, and v-region genes.

![Diagram](image)

Figure 4-1: Scheme of amplification of microdissected tissue

Microdissected tissue was extracted overnight, and as shown in Figure 4-1, samples were aliquoted into tubes, and amplified using a Qiagen OneStep RT PCR system, for detection of actin, insulin, or V-region genes as shown in Table 2-4. V-region primers are as shown in Table 2-4 actin and insulin primers are shown below, in Table 4-2.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Size of product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β ActinF</td>
<td>GGCCAACCGCGAGAAGAT</td>
<td>100</td>
</tr>
<tr>
<td>β ActinR</td>
<td>CGTCACCCGAGTCCATGA</td>
<td></td>
</tr>
<tr>
<td>Insulin primer 1</td>
<td>TGGCTGGGCTCGTGAAGCAT</td>
<td>115</td>
</tr>
<tr>
<td>Insulin primer 1R</td>
<td>GCCATGGCAGAAGGACAGTG</td>
<td></td>
</tr>
<tr>
<td>Insulin primer 2</td>
<td>TGTCTCTCTCCATGGCCCT</td>
<td>92</td>
</tr>
<tr>
<td>Insulin primer 2R</td>
<td>TTCACAAGGCTCGGCTGG</td>
<td></td>
</tr>
<tr>
<td>Insulin primer 3</td>
<td>CCCTGCAGAAGCGTGGCATT</td>
<td>133</td>
</tr>
<tr>
<td>Insulin primer 3R</td>
<td>CCATCTCTCTCGGTGCAGGA</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-2: primers used for amplification of insulin and actin (insulin from (Evans-Molina, Garmey et al. 2007), actin from Dr Kerry McLaughlin).

For V-region genes, RT-PCR using leader forward primers, with reverse hinge primers (Table 2-4 1 and 2) was followed by two sequential nested PCR reactions performed using GoTaq G2 (Promega, UK), set up as shown in Table 2-2, and cycled as shown in Table 2-3, using forward leader primers to nested reverse primers (Table 2-4 1 and 4), then inner forward primers to nested reverse primers (Table 2-4 3 and 4), resulting in 140 cycles of PCR. A schematic representation of this method is shown below in Figure 4-2, demonstrating the nesting of primers within the IgG sequence.
The reactions were analyzed in a 1.5% gel, and any visible bands or smears of appropriate size (200-300bp) were excised, purified using the BioRad Freeze N’ Squeeze system, and re-amplified using fully nested primers. PCR products were ligated into either pGEM-T-easy (Promega) or pCRII-TOPO (Life technologies).

**4.2.2 Ligation Reactions**

Ligation into pGEM-T-easy was performed using a 3:1 ratio of vector DNA to insert. Reactions were set up as in Table 2-5, and incubated for 1 hour. Ligation into pCRII-TOPO was performed according to the manufacturer’s instructions: reagents were assembled as shown in Table 4-3, and mixed for 20 minutes at 22°C.
Table 4-3: reagents for ligation into pCRII-TOPO vector.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product</td>
<td>2µl</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1µl</td>
</tr>
<tr>
<td>Water add to a total volume of</td>
<td>5µl</td>
</tr>
<tr>
<td>TOPO® vector</td>
<td>1µl</td>
</tr>
</tbody>
</table>

Ligated products were transformed into chemically competent XL1B cells, expanded overnight, and plasmids subsequently purified using the BioRad Quantum prep plasmid prep kit, according to the manufacturer’s instructions.

Purified plasmid DNA was measured using a Nanodrop device, and sent off for sequencing (Source Bioscience, Cambridge, UK). Sequences were analyzed against the VBASE2 database (www.vbase2.org), to determine if they were immunoglobulin sequences. As the primer pairs used here only amplify rearranged IgG, there can be no amplification of unrearranged, genomic V-region segments from non B cells.
4.3 Results:

4.3.1 Detection of B cells in inflamed islets and pancreatic lymph nodes on sections of pancreas from Type 1 diabetic patients:

In order to determine if there was any B cell infiltration in the pancreas samples available, it was necessary to visualize the presence of B or T cells in the pancreas, using immunohistochemistry: this also allowed islets with large areas of inflammation to be picked.

As seen in Figure 4-3 both lymph nodes and pancreatic islets of the type 1 diabetic pancreas from Oxford (OX0039) contains infiltrating B cells. The concentration of B cells was higher in lymph nodes, particularly in the germinal center. Sections were taken from samples across both the head and the tail of the pancreas OX0039: only some islets showed evidence of islet infiltration, and some islets remained insulin positive. Such heterogeneity is expected, as Walker et al showed evidence of islets with normal numbers of β-cells, and islets which responded normally to glucose stimulation (Walker, Johnson et al. 2011).
Figure 4-3: Immunohistochemical analysis of CD3 and CD20 expression in islets and follicles. A shows a follicle from a lymph node from recent onset type 1 diabetic pancreas, stained with anti-CD20 (DAKO). B. shows an islet from the same pancreas, stained for CD20 (brown). C. shows CD20 staining in the lymph at x40 and D. shows CD3 staining for T cells in the lymph node.

Samples from two more recent onset type 1 diabetic pancreases were obtained from Exeter. Islets and lymph nodes were microdissected by Pia Leete. Samples were transported on ice, in Qiagen buffer EB. Samples were extracted as before, and subjected to three rounds of PCR for β actin, VH and VL genes. Only one band was produced for the peripheral lymph node samples: as seen in Figure 4-13. The lymph node which generated a product is shown below in Figure 4-4, stained for CD20 in brown.
Figure 4-4: E581 PLN1 stained for CD20 (brown) staining and photography performed by Dr. Pia Leete.

To facilitate picking islets, insulin staining of adjacent sections was performed by Pia Leete, on samples from Exeter, as seen in Figure 4-5. There was no visible insulin staining in sample 039, therefore islets were visualized using contrast for this sample.

Figure 4-5: Insulin staining of pancreas Sc115, insulin in red. Islet 6 circled. Staining and photography performed by Dr. Pia Leete
4.3.2 RT-PCR amplification of β actin and insulin transcripts from diabetic and control fixed pancreas samples:

In order to determine if any intact mRNA remained in archival paraffin embedded samples, RNA was extracted from whole sections, and a 100bp product from β actin mRNA was amplified. PCR for β actin was successful in 9 out of 10 samples. Only Sample 039 section 2 failed to yield β actin transcripts, demonstrating that intact mRNA remains in the samples.

![Figure 4-6: Amplification of β Actin (100bp) from RNA taken from whole sections of diabetic (039) and control (080) pancreases. Only one section failed to yield β actin transcript.](image)

A 100bp section of actin transcript could be reliably amplified from RNA extracted from whole sections, as seen in Figure 4-6. Success of amplification was variable, but probably unrelated to the duration of storage, as both control and diabetic samples had been stored for 30-35 years.

![Figure 4-7: Amplification of two different insulin PCR products and a 100bp from actin mRNA. Transcripts were generated from recent onset Type 1 diabetic (039-1, 039-2) and healthy control (080-1) samples. Only insulin 2 could be amplified from sample 080-1. Expected product sizes were insulin (ins 1) and actin at 115bp, ins2 at 92bp and ins 3 at 113 bp.](image)

Amplification of actin and insulin from whole sections of pancreas is shown in Figure 4-7. Two sets of primers were used to insulin transcripts from RNA extracted from whole sections. As with β actin, success of amplification varied between sections and samples. Recovery of all products from the
control sample was lower than that of the recent onset diabetic sample, possibly due to poorer preservation of the control sample in this case.

![Figure 4-8](image)

Figure 4-8: Amplification of β actin from RNA extracted from whole pancreas sections without or with proteinase K treatment. The figure shows extraction without proteinase K treatment (1-4) and with proteinase K treatment (5-10).

A preliminary study looking at the use of proteinase K treatment to extract RNA/DNA showed no benefit in control human islets. As shown in Figure 4-8, β Actin transcript was obtained from 4/4 islets not treated with proteinase K and 1/7 treated with proteinase K. In further experiments, extraction was therefore performed in the absence of proteinase K.

![Figure 4-9](image)

Figure 4-9: Amplification of actin and insulin gene products amplified from a dissected islet, and exocrine tissue from a control subject (A56). Insulin products of the correct size were not amplified from the exocrine tissue.

Amplification of actin (spanning rearranged RNA) and insulin (1 covering intronic gDNA, 2 and 3 exons) seen in Figure 4-9, shows products for insulin (ins 1) and actin produced at 115bp, ins2 at 92bp and ins 3 at 113bp. No insulin products were seen in the exocrine tissue sample, however, both samples generated 100bp actin products, indicating that intact mRNA was present in the samples.

### 4.3.3 Amplification of immunoglobulin variable heavy and light chain fragments from inflamed islets and pancreatic lymph nodes from Type 1 diabetic patients

As intact mRNA remained in the archival samples, the methods developed in chapter 2 and 3 were adapted in order to attempt to amplify variable region IgG sequence from inflamed type 1 diabetic islets and peripheral lymph nodes (PLNs). Islets were dissected from a recent onset type 1 diabetic pancreas, OX0039, and peripheral lymph nodes taken at the same time. Tissue was extracted, and
amplified using pooled VH, VL and VK immunoglobulin leader primers, with hinge region reverse primers, according to the scheme shown in Figure 4-2. Pooled VH leader primers were subjected to a separate reaction to pooled light chain leader primer reactions.

**Figure 4-10:** Amplification of actin, insulin and immunoglobulin mRNA products, using mixed primer pools, on recent onset type 1 diabetic lymph node and pancreatic islets.

Analysis of RT-PCR products from amplification of islet and lymph nodes for actin, insulin and variable region genes showed that only 4 out of 16 islets from the recent onset diabetic sample produced bands for actin, two of which can be seen in Figure 4-10. The variable region PCR using mixed leader primers yielded a smear and clear 100bp actin bands for both PLN1 and islet 3. No samples produced visible products for insulin. The VHVLVK leader mix amplification smears were excised, gel purified and reamplified, using nested VH or VKVL forward primers, with hinge region reverse primers.

**Figure 4-11:** Amplification on heavy chain immunoglobulin, using mixed VH semi nested primers. Expected product was ~250bp.

Following the semi nested PCR, a product was seen in one lymph node sample, PLN1, which is shown in Figure 4-11. The band was excised from the gel, and a 2µl sample taken from the PCR reaction. Products were reamplified using single nested VH primer pairs, with nested reverse primers, producing the smallest IgG products, which are approximately 200bp. The results of the third, fully nested PCR are shown in Figure 4-12.
Specific nested PCR 2, again using single nested primer pairs, produced products from the pancreatic peripheral lymph nodes; the purified band from PCR2 (top) shows clear 200bp bands using the forward primers 1B, 3D, 4A, 4B and 798. The PCR product sample shows laddered bands with primers 2A, 4A, 4B and 789. All appropriate bands were taken and ligated into the pCRII-TOPO vector for sequencing.

Dissected islet and PLNs from Dr. Pia Leete in Exeter were subjected to a modified three step PCR reaction; in the final PCR reaction, samples were amplified using pooled nested VH primers, as opposed to the single primer pairs used on the OX0039 samples; this allowed fewer agarose gels to be used for analysis. PLN samples were amplified from both E581 and Sc115, however, only PLN1-C from the Exeter samples generated appropriately sized products. As can be seen below in Figure 4-13, a 220bp band was produced in the fully nested third round of PCR, and this band was excised, purified and ligated into pCRII-TOPO for sequencing. No bands could be seen in islet amplifications from this sample.

Figure 4-13: Amplification of immunoglobulin heavy chain from a pln from sample E581. A 220bp band was amplified from E581 pln1-C. This band was ligated into a TOPO vector, for sequencing. No appropriate size bands were generated from islet material from this section.

4.3.4 Amplification from islet material:
Twenty islets were extracted and amplified with VH primers, from the Oxford sample, 039. No appropriate bands were produced. Forty five islets were subsequently amplified using only VH primers, using three round of semi nested PCR, from samples received from Exeter. Of these, several
islets produced faint bands, which failed to improve with reamplification. One product, from islet 6, sample Sc115 (Sc115-6), could be amplified enough to attempt ligation. This product is shown in Figure 4-14 alongside more negative reactions from islets from sample E581. Although the band was nearly 300bp, which is larger than expected, it was purified and ligated into pCR II-TOPO for sequencing.

![Figure 4-14: Amplification of immunoglobulin heavy chains, using RNA from islets, with mixed VH primers. Only a faint band is visible for islet 6 (sc115-6) indicated by arrow, however this band is larger than expected for this reaction, at 300bp.](image)

### 4.3.5 Immunoglobulin transcripts generated:

Two bands extracted from the PLN of sample OX0039, PLN1 and PLN4, generated immunoglobulin heavy chain sequence; two heavy chain sequences were obtained from PLN1 and one from PLN4. Analysis of the two chains amplified from OX0039 PLN revealed all three sequences were derived from the VH3 gene family. The Exeter PLN sample, PLNC, generated one heavy chain sequence, also from the VH3 gene family. Sequences are shown below in Table 4-4.
Table 4-4: Immunoglobulin heavy chain sequences generated from PLN samples, with predicted protein sequences.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLN1A/B</strong></td>
<td></td>
</tr>
<tr>
<td>DNA: GAGGTGCAGCTGGGAGTCTGGGAGCCGCTCCATTAGACTCTCTCTGTCAG CCTCTGGTTTCACCTTCCAGTAAACGCCTGGATGAACTGGTTCCCGAAGCTCCAGGGAAGGGGCTGGAGTG GGTGGCCGTATTAAAGCAAAACTGATGGGACACGCTGCACCCGTGAAAGGCAGATTC ACATTCTCAAGAGATGGAACACGCTGTATCTGCAAATGA</td>
<td>EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAMNWRQAPGKLEWVGRKSTGDGGTDDYAAPVKGRFTI SRD DSKNTLYQMNSKTEDTAYYCTTDRDYVWNYGMDVWGQTTVTSS</td>
</tr>
<tr>
<td><strong>PLN4</strong></td>
<td></td>
</tr>
<tr>
<td>DNA: GAGGTGCAGCTGGGAGTCTGGGAGCCGCTCCATTAGACTCTCTCTGTCAG CCTCTGGTTTCACCTTCCAGTAAACGCCTGGATGAACTGGTTCCCGAAGCTCCAGGGAAGGGGCTGGAGTG GGTGGCCGTATTAAAGCAAAACTGATGGGACACGCTGCACCCGTGAAAGGCAGATTC ACATTCTCAAGAGATGGAACACGCTGTATCTGCAAATGA</td>
<td>EVQLLESGGGVLKPGRSLRLSCAASGFTSSYGMHWRQAPGKLEWVAISYDGTNKYYADSVKGRFTISRD NSKNTLYQINSRLAEDTAAYYCAKDRGLVVAATDYWGQTTVTSS</td>
</tr>
<tr>
<td><strong>PLN1C</strong></td>
<td></td>
</tr>
<tr>
<td>DNA: GAAATGCAGCTGGGAGTCTGGGAGCCGCTCCATTAGACTCTCTCTGTCAG CCTCTGGTTTCACCTTCCAGTAAACGCCTGGATGAACTGGTTCCCGAAGCTCCAGGGAAGGGGCTGGAGTG GGTGGCCGTATTAAAGCAAAACTGATGGGACACGCTGCACCCGTGAAAGGCAGATTC ACATTCTCAAGAGATGGAACACGCTGTATCTGCAAATGA</td>
<td>EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAMNWRQAPGKLEWVGRKSTGDGGTDDYAAPVKGRFTI SRD DSKNTLYQMNSKTEDTAYYCTTDRDYVWNYGMDVWGQTTVTSS</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
</tr>
</tbody>
</table>
Comparison of the sequences generated with germline sequences using V-Base 2 showed that all three sequences from OX0039 originate from separate germlines. The oligoclonal nature of these VH sequences suggests a relatively homogenous immune response to islet antigens in this PLN. Both PLN1a and PH4A from sample OX0039 have identical J segments. The sequence from E581 also uses the same junction segment.

Table 4-5: Analysis of germline, junction and diversity matches for sequences generated.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Germline Match</th>
<th>Junction match</th>
<th>Diversity Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>039-PLN1A/B</td>
<td>humIGHV178</td>
<td>X86355 IGHJ6*02</td>
<td>X93614 IGHJ3-16*01</td>
</tr>
<tr>
<td></td>
<td>humIGHV025</td>
<td>J00256 IGHJ6*01</td>
<td>X97051 IGHJ5-18*01</td>
</tr>
<tr>
<td></td>
<td>humIGHV271</td>
<td>X86356 IGHJ6*03</td>
<td>X13972 IGHJ5-5*01</td>
</tr>
<tr>
<td></td>
<td>humIGHV281</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>humIGHV267</td>
<td></td>
<td></td>
</tr>
<tr>
<td>039-PH4A</td>
<td>humIGHV195</td>
<td>X86355 IGHJ4*02</td>
<td>X97051 IGHJ1-26*01</td>
</tr>
<tr>
<td></td>
<td>humIGHV047</td>
<td>J00256 IGHJ4*01</td>
<td>X13972 IGHJ5-12*01</td>
</tr>
<tr>
<td></td>
<td>humIGHV199</td>
<td>M25625 IGHJ4*03</td>
<td>J00234 IGHJ2-15*01inv</td>
</tr>
<tr>
<td></td>
<td>humIGHV050</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>humIGHV029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>039-PLN1C</td>
<td>humIGHV242</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td></td>
<td>humIGHV041</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>humIGHV277</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>humIGHV096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>humIghv274</td>
<td>humIghv241</td>
<td>X86355 IGHJ4*02</td>
<td>M35648 IGHD2-2*03inv</td>
</tr>
<tr>
<td>------------</td>
<td>------------</td>
<td>-------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td></td>
<td>humIghv048</td>
<td>M25625 IGHJ4*03</td>
<td>X97051 IGHD2-2*02inv</td>
</tr>
<tr>
<td></td>
<td>humIghv240</td>
<td>J00256 IGHJ4*01</td>
<td>J00232 IGHD2-2*01inv</td>
</tr>
<tr>
<td></td>
<td>humIghv025</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>humIghv178</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Alignment of protein sequences show broad similarities on constant regions, as expected, since all sequences amplified derive from the same VH3 superfamily of heavy chains. However, CDRs, which determine antigen binding, are different.

### E581 sequence Alignment with 039 PLN VH sequences

<table>
<thead>
<tr>
<th>Sample</th>
<th>CDR1</th>
<th>CDR2</th>
<th>CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLN1A/B</td>
<td>GFTFSNAW</td>
<td>IKSKTDDGTT</td>
<td>TTDRYDYYVNYGMD</td>
</tr>
<tr>
<td>PLN4</td>
<td>GFTSSYG</td>
<td>ISYDGTNK</td>
<td>AKDRGLVVAATFDY</td>
</tr>
<tr>
<td>PLN1C</td>
<td>GFTSSYW</td>
<td>INSDGSST</td>
<td>AR</td>
</tr>
<tr>
<td>PLNC</td>
<td>GSFSFYSA</td>
<td>IRSRANSYAT</td>
<td>STGNWYPQY</td>
</tr>
</tbody>
</table>

One islet generated a band strong enough to ligate and sequence; Blast-n results show a match with
Homo sapiens MPN domain containing (MPND), transcript variant 2, as shown in Figure 4-15 and a less confident match with a B cell lymphoma product from chromosome 14.

![Blast results for sequence generated from amplification of islet material, showing a match with the MPND transcript variant 2.](image)

Figure 4-15: Blast results for sequence generated from amplification of islet material, showing a match with the MPND transcript variant 2.

The preserved VH gene repertoire of human peripheral B cells from pancreatic draining lymph nodes was analyzed using PCR analysis of individual germinal centers. A strong bias towards VH3 heavy chain family members was revealed. Amplification of light chains for all samples was unsuccessful: no light chain sequence was generated from any of the samples, despite repeated attempts.

4.4 Discussion:

This study examines whether transcripts representing IgG variable regions expressed by B cells in the diabetic pancreas can be isolated from archival tissue from both type 1 diabetic and control samples. In the two previous sections of this study, a mixed degenerate set of primers were used to amplify IgG variable region sequences for studies on the specificity of the immune response in diseased tissue: In Chapter 2 sequences were generated from mRNA extracted from frozen B cell lines, and in Chapter 3 from single IA-2 reactive B cells. In order to ascertain the feasibility of amplifying any material from preserved material, mRNA was extracted from whole sections, and a 100bp sequence representing part of the actin mRNA was amplified from several control and diabetic samples, both preserved for up to 30 years in paraffin. These results suggested it was worthwhile continuing with amplification of more complex targets, islets were microdissected from control sections and both actin and insulin sequences generated from these templates. Successful amplification of these targets from the majority of template sequences lead to the adaptation of techniques used in
previous chapters to generate sequence for IgG variable regions from archival islets and PLNs microdissected from tissue sections. Whilst the same technique was used for this study, however, the methods had to be adapted to compensate for the possible low levels of template DNA, by adding a third stage to the nested PCR approach. This strategy proved to be effective for amplification of template from lymph nodes, where template availability should be much higher than in individual islets, due to the presence of B cell rich germinal centers.

Although no IgG sequence was generated from islets, further testing of conditions of PCR and mRNA extraction may yet yield IgG sequence. The lack of sequence generated in this study may be due to more rapid tissue autolysis in the pancreas, or degradation of the low levels of B cell mRNA available; Willcox et al (Willcox, Richardson et al. 2009) suggest that up to 100 B cells can be counted in sections of infiltrated islets; whilst significant, there are likely to be higher numbers of B cells in dissected lymph nodes. In addition to this, not all dissected islets were likely to be infiltrated, as suggested by Willcox et al (Willcox, Richardson et al. 2009) where B cell infiltration was present, the mean minimum was 1·6 ± 0·3 (cells in moderately insulin rich islets-category 4) to a maximum of 21 ± 4·7 cells/islet section (cells in insulin low islets, category 2). Amplification of samples from both centers was successful, although the lack of success in generating any light chain sequence is one of the major limitations of this study. Amplification of light chains has been more challenging than that of heavy chains in chapters 2 and 3. Possibly generation of cDNA at lower temperatures would have increased the chances of amplifying both kappa and lambda chain sequences.

As with other studies, we used xylene to remove paraffin wax, and did not use RNase inhibitors in the assay. This method is optimized for specific tissues: the lymph nodes may be better preserved than other tissues. During the process of fixation and paraffin embedding, RNA will be degraded. However, enough RNA must remain intact for amplification, and using a mix of primers, with several round of PCR can only help to raise the chances of amplification of single intact transcripts remaining. Both the sizes of transcripts (110 bp for actin and insulin, and 320 bp for VH genes) and sequences generated support the concept that some amplifiable RNA remains in some archival tissues, despite overall degradation.

Samples used in this study were sourced from control and diabetic patients, collected before 1981. Sections were taken from 3 patients with established B cell infiltration of pancreatic islets. Several of these sections also contained fixed peripheral lymph nodes, with clearly defined germinal centers, which stained positive for CD20. PCR products of appropriate sizes were obtained from islets and lymph nodes of all three patients and three different VH gene transcripts were cloned from lymph node follicles of two patients. All three were of the VH3 gene family, and a bias towards
amplification of heavy chains from this family was also noted by Brezinschek et al (Brezinschek, Brezinschek et al. 1995) and two of the chains had identical junction segments: although there are 44 Variable, or V segments and 27 Diversity (D) segments, there are only six possible 6 Joining (J) segments. This means that the likelihood of encountering the same J segment is much higher than that of encountering the same D or V, explaining why the same J segment was seen in two of three heavy chains generated in this study.

This study demonstrates the feasibility of generating transcripts from archival tissue, for detailed analysis of specificity of immune responses within the Type 1 diabetic pancreas, even from samples stored for >30 years, although generation of heavy and light chain pairs for the generation of monoclonal antibodies is problematic due to the possible heterogeneous nature of the B cells in the infiltrate. However, it may be possible to determine the specificity of orphan heavy chains, using either irrelevant light chains or generating nanobodies constructed of homodimeric heavy chains (Wesolowski, Alzogaray et al. 2009; Hassanzadeh-Ghassabeh, Devoogdt et al. 2013; Muyldermans 2013). With the advent of nPOD, and the development of a library of fixed pancreatic tissue in the UK, the number of possible downstream uses for archival tissue has expanded. Retrospective analysis of pancreatic tissue from diabetic patients might permit a greater understanding of disease processes. The study of VH gene expression in the pancreatic lymph nodes widens our understanding of what is possible.
Chapter 5  Using NMR to visualize antibody binding of the IA-2 PTP molecule

5.1 Introduction:
Many epitopes for IA-2 autoantibodies are conformational; the central region epitope for the 96/3 monoclonal IA-2 antibody lies close to common autoantibody epitopes. Several studies have been carried out to map epitopes within the cytoplasmic domain of the IA-2 molecule (amino acids 601–979) using site directed mutagenesis, deletion mutants and phage display (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009; McLaughlin, Gulati et al. 2014; McLaughlin, Richardson et al. 2015). Epitopes within this region are diverse and antibody reactivity to several of these is inhibited by substitutions of cysteine 909 in the PTP domain, that represents a catalytic cysteine in active protein tyrosine phosphatases (Elvers, Geoghegan et al. 2013). Epitopes within the cytoplasmic domain of IA-2 include the JM domain (Lampasona, Bearzatto et al. 1996; Bearzatto, Naserke et al. 2002), conformational epitopes in the PTP domain requiring an intact C terminus (Dromey, Weenink et al. 2004; McLaughlin, Gulati et al. 2014) and a defined epitope within the central region (795–889) of the PTP domain (Zhang, Lan et al. 1997; Kolm-Litty, Berlo et al. 2000; Seissler, Schott et al. 2000). The monoclonal antibody 96/3 recognizes a conformational epitope in the central region, including the E836 amino acid of the IA-2 PTP domain, and competes effectively for binding with antibodies in many patient sera (Kolm-Litty, Berlo et al. 2000; Dromey, Weenink et al. 2004). Whilst some structural binding studies have been performed on this epitope, the specific conformation remains unclear; as both site directed mutagenesis and phage display may potentially cause conformational changes which could disrupt binding.

Both solution NMR and X-ray crystallography are commonly used to identify the structure of proteins and investigate protein-protein interactions (Yee, Chang et al. 2002). X-ray crystallography is the most commonly used technique to elucidate protein structures, in particular for proteins and complexes greater than 35kDa. Recent innovations in isotope labeling, combined with the availability of higher strength magnetic fields, with improved pulse sequence design have made the application of NMR for larger protein structure determination more commonplace. NMR has proved particularly useful for analyzing protein interactions, and proteins in living cells (Zuiderweg 2002; Sakakibara, Sasaki et al. 2009).

Although improvements have transformed the applications of protein NMR spectroscopy, use of this technique on larger proteins is still limited. Whilst X-ray crystallography can produce a 3 dimensional model of the structure of a molecule in hours, it does require the molecule to be
crystallized, which can be extremely difficult and time consuming. NMR in contrast can be performed on proteins in solution, or even within the cell, allowing analysis of native conformation and interactions. One of the most important limitations of NMR is the size of the molecules under investigation: NMR is best suited to proteins of less than 250 amino acids, meaning that any antibody-antigen complex to be analyzed should ideally be relatively small. Analysis and interpretation of NMR spectra can prove to be lengthy, and may not be ideal if the protein aggregates in solution or degrades quickly over time. Several crystal structures of the IA-2 PTP domain already exist (Almo, Bonanno et al. 2007; Kim, Jeong et al. 2007), however, none exist of the antigen complexed to antibody and the only information on antibody contact residues is derived from site directed mutagenesis, which may be misleading if mutations disturb protein conformation. We therefore propose to investigate changes in structure and identify the specific residues involved in forming the epitope recognized by a monoclonal antibody 96/3 using established NMR methods (Paterson, Englander et al. 1990). The 96/3 antibody recognizes a well characterized conformational epitope region of the PTP domain of IA-2 making this an ideal interaction to clarify structurally.

5.2 Using NMR to understand binding of the central region epitope:
The central region epitope of the IA-2 molecule, recognized by several monoclonal antibodies, including two IA-2 specific recombinant IA-2 antibodies generated as described in chapter 3, and a large proportion of patient sera (Dromey, Weenink et al. 2004), is well defined. This made this epitope a useful target region to test the feasibility of using NMR to map autoantibody epitopes on the molecule. Due to the polyclonal nature of patient sera, it is necessary to use previously generated monoclonal antibody fragments to specific regions of the IA-2 molecule to map specific epitopes.

Antibodies bind epitopes on their respective antigens by using six variable loops (complementarity defining regions or CDRs), which can bind epitopes on the target antigen with extremely high affinity. These loops fall within the variable region of the antibody molecule, and high affinity binding is generated by somatic hypermutation.

Understanding which epitopes are recognized in disease is important for improving our understanding of antibody-antigen interactions, which can lead to the development of neutralizing antibodies as valuable pharmaceutical agents (Shah, Foreman et al. 1994; Petit, Rak et al. 1997; Corti and Lanzavecchia 2013). Understanding epitope structure is also essential for the development of vaccines. Standard vaccines involve injecting an individual with target antigen in order to generate an antibody response. The ideal antigen for this purpose is a short peptide antigen (Ichihashi, Yoshida et al. 2011), identified by screening for immunoreactive epitopes. For immunotherapy of
type 1 diabetes, knowledge of antibody epitopes may assist in design of treatments to delete or inactivate autoantigen specific B cells, or characterize T cell epitopes participating in B cell/T cell collaboration.

The importance of NMR in the biophysical analysis is due to its capability of identifying binding sites, at the level of individual amino acids. The technique can also be used to elucidate structures of flexible proteins and low-affinity (Kd > 10-6 M) complexes, which can be difficult to crystallize. The essential concept is that the NMR signal of an antigen will change upon antibody binding, causing a chemical shift perturbation. To date, there are a large number (>1000) published studies using NMR to identify sites of protein-protein interactions.

Paterson et al (Paterson, Englander et al. 1990) studied monoclonal antibody binding to a relatively small molecule (Cytochrome C) with a previously assigned backbone, using immunoprecipitation of the antigen by the antibody with hydrogen exchange labeling using D2O followed by 2 dimensional 1H NMR analysis. By recording repeated COSY spectra over several months it was possible to determine which NH groups remained un-exchanged, and were therefore protected by antibody binding.

Addis et al (Addis, Hall et al. 2014) generated a series of 15N/1H and 15N/13C/1H two dimensional 1H,15N TROSY spectra for both free and IL-1β and IL-6-bound scFvs and Fabs, to determine sequence-specific backbone resonance assignments for the antibody fragments used. Observations of chemical shift perturbations on addition of antigen were performed using a minimal shift method, measuring perturbations in 15N, 13C, and 1H nuclei. This study demonstrated specific structural perturbations within distinct antibody fragments on binding of protein antigen, allowing clear definition of the antigen binding sites.

The Simonelli group used NMR to validate epitope predictions generated using computational docking. The group assigned a backbone to the DVIII protein using a series of HNCACB and 15N-NOESY-1H,15N HSQC experiments, followed by 1H,15N HSQC to generate a spectrum to visualize the unbound antigen followed be a series of spectra generated with both antigen and antibody; on addition of antibody, the local chemical environment of bound residues changes, causing a perturbation in the NMR signal. Identification of bound amino acids using the backbone assignment was used to validate binding of the antibody DV32.6 with dengue virus peptides (Simonelli, Pedotti et al. 2013).
These results suggest that it should be possible to measure chemical shift perturbations upon binding of a labeled PTP protein with 96/3 scFv. In order to determine the identity of bound residues, a backbone assignment of the PTP protein would be required.

5.3 Aims:
The aims of this section of the study were to assess the use of NMR to map conformational autoantibody epitopes within the PTP domain of the IA-2 molecule. In order to map chemical shift perturbations in amino acid binding, a backbone assignment would first be necessary. This study assessed the feasibility of both a backbone assignment and whether any chemical shift perturbations upon antibody binding could be visualized using NMR.
5.4 Methods:

5.4.1 PTP isotope labeling and sample preparation: Expression of PTP protein for NMR:

Table 5-1: Protein sequence of PTP, with GST tag cleaved using PreScission protease, expressed in pGEX-6P-1 (GE Healthcare)

<table>
<thead>
<tr>
<th>PTP protein sequence GST tag cleaved (33 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPLGSMILAYMEDHLRNDRDLAKEWQLACYQAEPNTCAAGGEENIKKNRHPDFLDPYDHARIKLKVESSPSRDYINASPIIEHDPMPAYATQGPLSHTIAADFWQMWWESGCTIVMLTPLVEDGKVQCDERYWPDEGASLYHVEVNVLVSEHIWCEDFLVRSFYLKTVQTQETRTLQFHFLSWPAEGTPASTRPLLDFRKRKNVKYRGRSCPIIVHCSDGAGRTGTYLLIDMVLNRMAKGVKEIDIAATLHEVRDFQRPGLVRSKDFEFALTAVAEVNLKALPQ**</td>
</tr>
</tbody>
</table>

A truncated version of the IA-2 protein, representing the protein tyrosine phosphatase domain of the protein, sequence shown in Table 5-1, had previously been generated in the expression vector PGEX-6P-1 (GE healthcare, Amersham, UK), shown in Figure 5-1. The pGEX-6P-1 vector generated protein with a PreScission protease (GE healthcare) cleavable GST tag for purification. In order to determine whether the protein folded correctly a 1D NMR spectrum was generated, using 500µl of IA-2 PTP protein at a concentration of 3.5mg/ml. Protein was dialed into PBS, and spiked with 10µl deuterium for acquisition of NMR spectra.

Figure 5-1: Map of the pGEX 6P-1 vector (GE Healthcare), used for expression of the IA-2 protein.
5.4.2 15N labeling for preliminary NMR analysis prior to analysis of chemical shift perturbation:

Protein was expressed using a commercial cell labeling medium (Celtone, Cambridge Isotopes) to generate labeled protein for 2D NMR. IA-2 PTP was expressed in E.coli strain BL21 (DE3). A 5ml LB/Ampicillin culture of IA-2 PTP was spiked to grow overnight, and added to 1L of Celtone media the next day. Labeling of protein was performed using Celtone commercial 15N labeled growth medium, for maximum protein yields. The cells were grown for 16 h at 37°C in Celtone rich medium isotope-enriched (Martek Biosciences Corp., US), containing ampicillin (100μg/ml). After OD 600 reached 0.6, cells were incubated for 45 min at 30°C, induction was performed by adding 1mM isopropylthio-β-D-galactoside (IPTG), and expression performed for 6–8h at 30°C. Cells were spun down at 10000rcf, and pellets resuspended in PBS containing PMSF (100μg/ml), Benzamidine (1mM) and DTT (0.5mM). Pellets were lysed in the presence of lysozyme, and DNA removed by DNAse treatment. Lysate was rolled with 1ml/L culture anti-GST Sepharose 4b beads (GE Healthcare) for 4 hours at 4°C, washed 3x in PBS/PMSF, and applied to a disposable column. The column was then equilibrated in PreScission protease cleavage buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM DTT, pH7.0) and drained. PreScission protease (80 Units in 160µl cleavage buffer, per 2L of culture) was added to the column, and incubated overnight at 4°C. PreScission Protease is a fusion protein, consisting of human rhinovirus 3C protease with a GST tag. The tagged protease cleaves between the Gln and Gly residues of the recognition sequence of Leu Glu Val Leu Phe Gln/Gly Pro, whilst the GST tag ensures that the protease remain bound to resin. Protein was eluted the next day in 1ml fractions of cleavage buffer. PreScission protease remains bound to Sepharose 4B resin along with the remaining section of the GST tag. Protein purity was analyzed by gel electrophoresis and protein concentration by Bradford assay. Protein-containing eluates were pooled and dialyzed into PBS/PMSF. A typical yield of ~4 mg of 15N labeled IA-2 PTP per L of Celtone medium was obtained.

Protein-containing aliquots were pooled, dialyzed into PBS and concentrated using Amicon ultra 10K cutoff spin concentrators (Millipore, UK), spinning at 3000rcf. An 80µM sample was prepared and placed in a 5mm Shigemi tube (Shigemi Inc., US) for NMR analysis.

5.4.3 2H,15N labeling for assessment of chemical shift perturbations

IA-2 PTP protein was once more generated by expression in BL21 (DE3) cells. Labeling of protein was performed using Celtone commercial 2H,15N, labeled growth medium, for maximum protein yields. The cells were grown and harvested as before, and protein purified in the same manner. Unfortunately, yields of intact protein were much lower in the double labeled medium (1mg/3L culture).
Samples were concentrated using Amicon ultra 3K cutoff spin concentrators (Millipore, UK), to limit losses. A 70µM sample was prepared and placed in a Wilmad NMR tube (Wilmad Labglass, New Jersey USA) that facilitates the addition of scFv when analyzing chemical shift perturbations after formation of antibody-antigen complexes.

**5.4.4 Expression of 96/3 scFv:**

The 96/3 scFv was prepared as in Chapter 2. Briefly, the 96/3 scFv was expressed with a hexa-histidine tag using the pAK19 vector, with a low phosphate induction. The 96/3 scFv yielded on average 2mg/L culture. The scFv was purified using NiATA agarose (His Select resin, Sigma, UK), and eluted with 300mM imidazole. Fractions containing protein were analyzed by Coomassie staining and by anti-His tag Western blotting, using a biotinylated anti-penta His antibody (RnD systems, Abingdon, UK). His-tagged protein was then dialyzed into PBS, and stored at 4°C.

**5.4.5 Coomassie staining and Western blotting:**

10µl samples of protein (lysate and eluates) were added to 5µl of 4x NuPage sample buffer (Invitrogen) and 3µl Dithreothreitol (DTT). Samples were mixed and heated to 90°C for 10 minutes, then loaded into a 12%bis/Tris gel (Invitrogen), in MES running buffer. Gels were run at 200V constant Voltage, for 45 minutes. Gel cassettes were then split to release gels. For Coomassie staining, gels were placed in Coomassie R-250 solution (2% Coomassie R-250, 10% acetic acid, 50% methanol), microwaved on full power for 30 seconds, then stained at room temperature for one hour. Gels were washed in tap water and destained overnight, in 10% acetic acid, 20% methanol, 70% H₂O.

For Western blots, gels were assembled into a sandwich with pre hydrated PVDF membrane, filter papers, and sponge padding. After hydrating the membrane in methanol, all components were soaked in transfer buffer (Invitrogen) to pre equilibrate. Gels were transferred for an hour at 30V. Transferred membranes were blocked in 5% milk, probed overnight with anti-GST (1:1000, ABD Serotec, Oxford, UK), anti-penta his TAG (1:1000, RnD systems) or M13 anti-IA-2 (1:1000, RSR, Cardiff). Membranes were washed then probed with HRP-conjugated secondary antibody (anti-mouse, goat or human IgG Fc specific (Sigma-Aldrich)) at a concentration of 1:1000 in TBS-T for 1 hour. Membranes were washed again and developed using 2% 3-amino-9-ethylcarbazole (AEC) in acetone, 2ml of which were diluted in 69ml H₂O, 2ml 3M sodium Acetate, pH5.5, 125µl H₂O₂, for 10 minutes at room temperature, followed by several washes in tap water.
5.4.6  **Bradford assay to determine protein concentration**

The Bradford (Thermo Scientific, UK) assay was used to determine protein concentration, as the presence of Glutathione S transferase (GST) does not interfere with this assay. 5μl samples of a BSA standard curve (10mg/ml-0.2mg/ml) were added to a Maxisorb plate (Thermo Scientific, UK), with 5μl samples of prepared protein (lysate or eluate). All samples were incubated with 200μl of Bradford assay reagent for 10 minutes, at room temperature. Absorbance was analyzed at A595, using a Chameleon plate reader (HideX, UK), and protein sample concentrations calculated against the standard curve.

5.4.7  **NMR spectroscopy:**

Solution NMR spectroscopy was used to analyze the resonance spectra of IA-2 PTP alone and after binding of the 96/3 scFv. ¹H,¹⁵N NMR spectra were recorded at 25°C on a Bruker Avance III spectrometer equipped with z-gradient triple-resonance probes and BTO temperature regulation units. All NMR analysis at Kings College London was performed by Dr. A. Atkinson. The spectra were processed with Topspin NMR software. NMR spectra on the doubly labeled ²H,¹⁵N protein were generated on a Bruker Avance III 950MHz spectrometer, located at the MRC NMR Biomedical Center at the Crick Institute, Mill Hill laboratories. The spectra were processed with Topspin NMR software. All NMR analysis at Mill Hill was performed by Dr. G. Kelly.
5.5 Results

5.5.1 Analysis of Protein folding by 1 dimensional NMR:

Figure 5-2: 1D ^1H NMR of unlabelled IA-2 PTP protein (120µM), performed at 25°C, at 700MHz, at 200µM in 100mM Tris/5mM EDTA buffer.

PTP protein was expressed in terrific broth (Sigma, UK), dialyzed in 100mM Tris/5mM EDTA buffer and concentrated using Amicon ultra 10K cutoff spin concentrators. 1D ^1H NMR spectra were recorded at 25°C on a Bruker Avance III spectrometer at a concentration of 200µM in 100mM Tris/5mM EDTA buffer. The spectrum is shown in Figure 5-2, with each peak in the spectrum representing a proton in the sample. As shown, the signals are broadly defined and spread over the chemical shift area, suggesting that the protein is well folded. In an unfolded protein the peaks would be much sharper and cover a smaller range of the spectrum (Page, Peti et al. 2005).

5.5.2 Determination of the optimum conditions for expression of the PTP domain of IA-2:

Expression levels of IA-2 PTP protein in standard Luria broth were relatively low (<2mg/L culture), necessitating optimisation of expression media in order to produce adequate quantities of protein for NMR. Protein was expressed in both NMR minimal media and a commercial broth, Celtone, in
unlabelled form. SDS-PAGE of lysate and affinity purified factions are shown in Figure 5-3. Minimal media expression yielded 0.8mg/L culture, whilst expression in Celtone gave 2.2mg/L culture, or a 2.4 fold increase in yield. More contaminants were also seen in the minimal media purification. Therefore, Celtone rich media was used in all further preparations of IA-2 PTP protein.

Figure 5-3: Coomassie stain comparing expression media (Celtone and LB). Showing A. Celtone unlabeled media, lys (lysate) FT (Flow through) and Purified (affinity purified protein) B. shows protein expressed in minimal media (lysate) FT (Flow through) and Purified (affinity purified protein). Untagged PTP at 28-32kDa (indicated by arrows) and GST tagged protein at 50kDa.

5.5.3 ¹⁵N labeling of PTP protein: ¹H,¹⁵N HSQC data:
In order to optimize expression levels, and determine optimal purification methods, IA-2 PTP was expressed with a ¹⁵N label. ¹⁵N labeling enables us to generate a two dimensional ¹⁵N/¹H plot with clear resolution of individual resonances for each amide group in the protein, in contrast with proton NMR only, which generates a trace where individual resonances cannot be distinguished. Purity of PTP protein was assessed using a Coomassie stain, shown in Figure 5-4. Whilst a total of 4.5mg was
generated from 3L of culture, upon concentration, much of the protein was found to have been lost in the spin concentration step.

Spectra were generated, but the signal-to-noise ratio was poor, due to the low concentration of the protein, and over the course of several days the protein degraded, most likely due to proteases in the preparation. Protease contaminants were likely to be present at low concentrations as very few bands were visible in the Coomassie stain (Figure 5-4).

![Coomassie stain of N^{15} labeled PTP; showing lysate, then eluates 1, 3, 5, 7 and 9. Protein is present in eluates 1-7. Bands run at a slightly higher Mr in this gel, possibly due to using non gradient gels for this assay.](image)

\textsuperscript{1}H, \textsuperscript{15}N HSQC spectra were recorded for IA-2 PTP. The low concentration of the protein caused an increase in background noise, as can be seen in the \textsuperscript{1}H,\textsuperscript{15}N HSQC spectrum shown in Figure 5-5. Isolated individual signals for amide group residues can be seen, but many signals in the center of the spectrum did still show some degree of overlap.
Within the $^1$H,$^{15}$N HSQC spectrum, boxed areas show probable indole side chain from tryptophan residues outlines in red. Protein was analyzed at both 25°C and 37°C; however, there was a visible loss of signal, suggesting aggregation, and degradation, shown by the appearance of new c-termini, in Figure 5-5B.

5.5.4 **Optimization of spin concentration of the antigen:**

To uncover the reasons for large loses of protein during purification and concentration, a fresh batch of 500ml culture of PTP protein was expressed, and purified as before. The total protein yield was 3mg/500ml culture volume. Protein was concentrated by successive centrifugation steps at 3000rcf in an Eppendorf benchtop centrifuge. 10µl samples were taken at ten minute intervals. On concentration, protein contaminants with Mr >40 000 were observed (Figure 5-6, indicated by red arrows). On reaching concentrations of 9.55mg/ml, the soluble protein concentration recovered from the spin concentrator decreased, and significant amounts of protein were visible in flow through (ft) fractions. Not all lost protein was accounted for by loss to flow through, however, suggesting protein is forming insoluble aggregates which accumulate in the spin filter. Concentration steps and flow through concentrations are shown in Table 5-2.
Figure 5-6: Coomassie stain assessing spin concentration of PTP protein. There is a large band between 30 and 40kDa believed to represent the PTP protein. Gels shows large amounts of 30-40kDa protein in flow through, and a drop in column protein concentration, identified using a Nanodrop device as being after protein concentrations reach 9.55 mg/ml. Arrows indicate high level contaminants.

Table 5-2: Progressive spin concentration of IA-2PTP protein; protein concentration begins to decrease at 9.55mg/ml. Flow through was completely removed each time, so each measurement represents fresh flow through.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Volume(µl)</th>
<th>Concentration (mg/ml)</th>
<th>Flow through concentration (mg/ml)</th>
<th>Protein yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2000</td>
<td>1.02</td>
<td>0.47</td>
<td>0.99</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>0.99</td>
<td>0.51</td>
<td>1.145</td>
</tr>
<tr>
<td>12.30</td>
<td>70</td>
<td>6.36</td>
<td>0.6</td>
<td>0.4452</td>
</tr>
<tr>
<td>22.30</td>
<td>30</td>
<td>9.55</td>
<td>0.6</td>
<td>0.28</td>
</tr>
</tbody>
</table>

5.5.5 Prevention of Aggregation:

As protein also showed visible signs of aggregation and precipitation over the period the NMR spectra were taken, optimization of buffers was undertaken to try and prevent protein loss over the period of the experiment. PTP protein was dialyzed into one of two buffers: 20mM phosphate or 20mM Tris/ 1mM EDTA/ 100mM NaCl. Protein was the stored at one of three temperatures over three days, and concentration of protein in solution measured using a Nanodrop. As can be seen in
Table 5-3, protein concentration was more stable in 20mM phosphate buffer than in Tris/EDTA but aggregation occurs in all samples at high concentrations, as seen by the drop in soluble protein concentration. Progressive loss of concentration is less visible in low protein concentrations. PTP protein for use in other experiments had been successfully stored at 4°C, over a period of weeks in PBS; therefore it was decided to run all future experiments in PBS.

Table 5-3: Testing aggregation of PTP protein over time by measuring decrease in concentration in two buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Starting Concentration (mg/ml)</th>
<th>4°C Concentration (mg/ml)</th>
<th>22°C Concentration (mg/ml)</th>
<th>37°C Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM NaPhosphate</td>
<td>3.24</td>
<td>2.98</td>
<td>2.56</td>
<td>2.49</td>
</tr>
<tr>
<td>20mM NaPhosphate</td>
<td>9.67</td>
<td>2.74</td>
<td>1.9</td>
<td>2.01</td>
</tr>
<tr>
<td>20mM TrisEDTA</td>
<td>3.15</td>
<td>2</td>
<td>2.01</td>
<td>1.2</td>
</tr>
<tr>
<td>20mM TrisEDTA</td>
<td>9.89</td>
<td>1.4</td>
<td>1.01</td>
<td>0.98</td>
</tr>
</tbody>
</table>

As protein both came through the filter, and aggregated at higher concentrations, it was deemed unusable for a backbone assignment without recloning to improve yields, and optimization of buffers to prevent aggregation. In order to assign a backbone to a protein of interest, a protein sample of a concentration of 300-500µM is required to generate NMR spectra essential for this process (Breukels, Konijnenberg et al. 2011). Unfortunately due to the tendency of the protein to aggregate, it was not possible to produce a stable protein sample at a concentration higher than 228µM (8mg/ml), preventing further attempts to perform backbone assignment of IA-2 within the time available for this project.

5.5.6 Concentration using PBS:

In order to test if a high salt buffer would improve concentration conditions by limiting protein accumulating in the spin filter, spin concentration of the PTP protein was performed in PBS buffer. Losses to flow through were decreased, and protein concentration was improved, as seen in Figure 5-7.
Figure 5-7: Graph showing spin concentration of PTP protein in PBS buffer; a continuous increase in soluble protein concentration can be observed, without evidence of aggregation.

5.5.7 Expression of 96/3 scFv:
The 96/3 scFv was expressed in 4L of culture. The final yield was 3.2mg/4L. The protein was run on a 12% Bis Tris gel, and analyzed by Coomassie staining. As can be seen in Figure 5-8, there is a strong band at 27kDa, representing the scFv, but several faint bands visible at 50 and 60kDa, which could be Heat Shock Proteins (HSP) as suggested by Bolanos Garcia et al (Bolanos-Garcia and Davies 2006).

The scFv prep was also analyzed by Western blot, detecting the penta his tag on the protein. The Western blot shows only the scFv band at 27.5kDa, seen in Figure 5-8 with no contaminants at 50kDa.
5.5.8 $^2\text{H},^{15}\text{N}$ labeling of IA-2 PTP for $^1\text{H},^{15}\text{N}$ TROSY and detection of chemical shift perturbations:

In order to determine if binding of the 96/3 scFv could be visualized using NMR, a $^2\text{H},^{15}\text{N}$ doubly labeled sample was expressed and purified. $^{15}\text{N}$ labeling allows better resolution of proteins (see Introduction sections 1.7, 1.8), as deuteration of proteins slows $^1\text{H}-^1\text{H}$ relaxation, giving a sharper signal for larger molecules that display fast relaxation rates (short T2 values). T2 is inversely proportional to the width at half-height of the NMR signal (Breukels, Konijnenberg et al. 2011), therefore short T2 values would correspond to broad line-widths.

Two extra column volume washes were incorporated into the purification, to try to eliminate protease and other contaminants. As can be seen in Figure 5-9, the protein produced was sufficiently pure, although at a low concentration with only 1mg of pure protein being produced from 3L of culture. Protein was concentrated, and a concentration of 65µM was achieved, with minimal losses during the concentration step. The protein was kept in solution in PBS, which is a high salt buffer for NMR purposes, but helped to prevent aggregation.
5.5.9 2D $^{1}H,^{15}N$ TROSY analysis of the IA-2 PTP domain:

The PTP domain of IA-2 is moderately large for NMR analysis (35kDa), which limits the resolution of spectral peaks generated using a single label (e.g. $^{15}N$): the T2 relaxation time of the nuclei in a protein decreases with an increase in protein size (Clore and Gronenborn 1991; Wagner 1993) and this causes line broadening with consequent loss of resolution and much worse signal-to-noise ratio.

For larger proteins however $^{1}H,^{15}N$ TROSY (Transverse Relaxation Optimized Spectroscopy) spectra can ameliorate these effects (Foster, McElroy et al. 2007), being a pulse sequence that takes advantage of the intrinsic relaxation pathways of $^{15}N$-$^{1}H$. $^{1}H,^{15}N$ TROSY experiments work better with deuterated samples. Therefore both $^{1}H,^{15}N$ HSQC and $^{1}H,^{15}N$ TROSY spectra were obtained for the PTP protein. To generate optimal $^{1}H,^{15}N$ TROSY data, it is considered useful to perform a heteronuclear label: using deuterium ($^{2}H$) (Riek, Fiaux et al. 2002).

In order to detect chemical shift perturbations caused by antibody binding using NMR, a spectrum of the unbound labeled protein must first be taken, then the antibody fragment added and another spectrum recorded. As the antibody fragment is unlabeled, it will not appear in the $^{15}N$-filtered spectrum (i.e. $^{1}H,^{15}N$ HSQC and $^{1}H,^{15}N$ TROSY), although it will be visible in the $^{1}H$ spectrum. Any bound residues should experience chemical shift perturbations between bound and free spectra, as the environment in which the residue finds itself has changed.
5.5.9.1 $^1$H, $^{15}$N TROSY:

$^1$H, $^{15}$N TROSY experiments were run on a Bruker 950MHz spectrometer located at the MRC NMR Biomedical Center at the Crick Institute, Mill Hill laboratories. These experiments were performed by Dr. Geoff Kelly. Spectra were recorded at 25°C. The initial $^1$H, $^{15}$N TROSY spectrum confirmed that the labeled PTP protein was stable over a period of 24 hours, and ~170 of the 293 residues could be seen, as shown in Figure 5-10.

![Figure 5-10: $^1$H,$^{15}$N TROSY spectrum of the PTP molecule, performed using the $^2$H$^{15}$N labeled IA-2 protein, at 25°C, in PBS buffer, at a concentration of 65µM. The images shows the whole spectrum, inset box outlining tryptophan indole NH side chain cross-peaks (bottom left).](image)

Figure 5-10 shows a 2D $^1$H,$^{15}$N TROSY spectrum of the PTP protein, recorded over five hours. The spectra generated show clearly defined separate peaks, with discernible residues. No degradation or aggregation was observed over the 24 hour period that the spectra were recorded for the PTP protein. The boxed area inset shows the probable location of the indole side chain NH-cross peaks of tryptophan residues: there are 6 tryptophan residues in the PTP molecule, and six peaks can be seen in this area, suggesting they may all be resolved.

The data generated in the initial $^1$H,$^{15}$N TROSY recording of the PTP protein suggest that optimization of purification and concentration of the PTP protein after the initial $^{15}$N experiments prevented degradation and increased the signal to noise ratio. Future experiments should focus on improving
expression levels and concentration methods to obtain adequate quantities of protein for a more detailed analysis.

Figure 5-11: $^1$H,$^{15}$N TROSY spectra of IA-2 PTP protein performed using the $^1$H$^{15}$N labeled protein over a time span of several hours, at 25°C, in PBS buffer, at a concentration of 65µM taken at 4 hour intervals A. after four hours, B. after eight hours, C. after 12 hours and D. after 16 hours.

Sequential spectra taken every four hours show that the PTP protein was stable under these conditions. No degradation could be seen in the protein (Figure 5-11).

5.5.10 Detection of chemical shift perturbations on complexing with 96/3scFv

In order to determine if binding of amino acids within the PTP domain could be visualized using NMR, labeled PTP protein was complexed with the 96/3 scFv preparation, at a molar ratio of 3:1 scFv to PTP protein. Ideally the scFv would have been added in a titration: moving from a low concentration of scFv to an excess, in order to allow maximum visualization of possible chemical shift perturbations occurring during binding. However, low yields of both proteins prevented a titration experiment. After addition of the scFv to excess, both 1D $^1$H spectra and $^1$H$^{15}$N TROSY spectra were recorded.
Figure 5-12: 1D $^1$H NMR of A. IA-2 PTP performed using the $^2$H$^{15}$N labeled IA-2 protein, at 25°C, (spectrum generated every 4 hours, in PBS buffer, at a concentration of 65µM. A shows PTP protein alone. In this figure, the exchangeable protons (NH groups) are clearly visible at low field, whereas there is a low signal in the rest of the spectrum arising from the small fraction of non-exchangeable protons represents the deuterated sample. B. shows spectra of $^2$H,$^{15}$N IA-2 PTP with the unlabeled 96/3 scFv. The increase in proton signals appearing show the addition of the unlabelled 96/3 scFv.

In Figure 5-12 A, the NMR spectra of IA-2 PTP protein shows that the protein remains well folded for the duration of the experiments on the protein alone seen in Figure 5-11 and Figure 5-12. In Figure 5-12B, on addition of the 96/3 scFv, the proton signals of the methyl groups show a well folded scFv (resonating at high field, on the right side of the spectrum), and these did not change over time, indicating that that the 96/3 scFv remained stable for the duration of the experiment and beyond.

Figure 5-13, however, shows the progressive degradation of the PTP sample. A. shows the initial intact sample. As time progresses following addition of the scFv fragment, the $^1$H,$^{15}$N TROSY spectrum shows the appearance of a number of resonances resonating around 8 ppm and all clustering towards the center of the spectrum. This is indicative of degradation of the PTP protein.
Figure 5-13: Showing $^1$H, $^{15}$N TROSY of initial sample, followed by subsequent spectra taken after addition of 96/3 scFv prep, demonstrating progressive degradation of sample. Samples were analyzed every 4 hours.

A subsequent Coomassie stain of the $^2$H, $^{15}$N PTP scFv NMR sample, Figure 5-14, shows a band at Mr 25, consistent with the presence of intact scFv, however, there are also higher molecular weight contaminants. In the gel, very little Mr 37 protein can be seen, confirming the NMR experiments that the PTP protein is degraded during the experiment, as suggested by the presence of multiple lower molecular weight bands, which may represent degraded PTP protein.
High level protease contaminants were clearly present in the scFv prep, and whilst they did not affect the scFv, they were able to degrade the PTP protein. This underlines the importance of removing all contaminants from both protein preparations to be used in these studies.

Nonetheless, chemical shift perturbations of specific residues were seen early after addition of 96/3 scFv, seen in Figure 5-15, and despite the concurrent beginning of PTP degradation which hinders the analysis, chemical shift perturbations upon scFv addition can be identified. This clearly suggest the feasibility of such studies and also suggest that with a better sample purification an epitope mapping of PTP may indeed be achieved.
1.5,15N TROSY spectrum recorded on addition of 96/3 scFv. A superposition of 1H,15N -TROSY spectra for PTP in the absence (blue contours) and presence (red contours) of 96/3 scFv. Insets show possible shifted residues (blue-red). Multiple new c-termini can be seen appearing, indicating degradation.

5.6 Discussion:

NMR has traditionally been used to determine protein structures, of smaller molecules without the need for crystallization. More recently, however, the study of protein–protein interactions by NMR has become a widespread application. This evolution became possible due to improvements in NMR equipment, development of more efficient isotopic labeling schemes, especially perdeuteration (Venters, Farmer et al. 1996), and the advent of 1H,15N TROSY (Riek, Wider et al. 1999; Pervushin 2000; Riek, Pervushin et al. 2000). Whilst NMR epitope mapping would have an obvious advantage over site-directed mutagenesis, as conformational epitopes are directly mapped, the technique suffers from clear disadvantages: the antigen needs to be labeled with NMR active nuclei, and larger antigens provide additional challenges, with both spectral overlap and signal broadening causing a loss of sensitivity. Assignment of amino acids to particularly peaks proves an even greater challenge in larger molecules: of the 10996 proteins in the protein database (PDB) with a backbone assignment, most are less than 20 kDa in size: only 29 of them above 40 kDa, demonstrating the difficulty of performing an assignment for a protein the size of the PTP domain of IA-2 at 35kDa. Whilst the 1H,15N TROSY spectrum of the PTP protein shows multiple distinct peaks, a backbone assignment for this protein would not be a trivial undertaking, especially as it was found to be
extremely difficult to prepare the recombinant protein construct at suitably high yields and concentrations. Generating a three dimensional structure from the NMR spectra would be an even greater undertaking.

However, the greatest obstacle to generation of spectra was generating protein of adequate purity: use of additional purification steps including size fractionation and ion exchange chromatography may have allowed clearer analysis of protein containing fractions, although potentially with reduction in yields. The 96/3 scFv was purified using nickel-agarose resin, which has been shown to bind proteins with multiple histidine residues, in particular heat shock proteins (HSPs); As shown by Robichon et al (Robichon, Luo et al. 2011), His-tagged proteins expressed in *E. coli* and purified by immobilized metal affinity chromatography are commonly co-eluted with native *E. coli* proteins, which display a high affinity for metal ion resins, due to the presence of clustered histidine residues, or relevant metal binding sites.

In order to identify the epitope recognized by the 96/3 scFv, it would be necessary to perform a backbone assignment for this protein. Due to the relatively low levels of expression of PTP protein and the degradation of the antigen on addition of the scFv fragment, with contaminating proteases, this was not possible. However it is clear that at low concentration, in PBS buffer the IA-2 protein folds well, and is stable at 25°C. Although concentrations of protein prepared in this project were not adequate for backbone assignment, the clear discrimination of individual peaks in the 1H,15N TROSY experiment suggests that NMR shift assays for epitope identification may be feasible if the problems of protein aggregation and degradation can be overcome. Although the addition of scFv to the complex caused degradation of the sample, changes in some nuclei were visible, suggesting that there were observable chemical shift perturbations which could be caused by antibody binding. Both an improvement in expression levels of the PTP protein, and production of a 96/3 scFv preparation free of contaminating proteases would greatly help future analyses of binding of the IA-2 PTP protein in NMR. Although some other studies have used longer peptides (Huth, Olejniczak et al. 2000), representing the target binding site of a protein to improve resolution and improve backbone assignment of spectra generated, this would not be advisable to the IA-2 protein as it is a globular domain, and smaller fragments are high unlikely to fold appropriately for antibody binding. Therefore, the solution may lie in generating a larger molecule which expresses at higher levels, possibly using codon optimization for maximum protein expression in *E. coli*. Higher expression levels would cut the cost of labeling and expression, facilitate backbone assignment and improve expression levels. Future work on this protein should optimize expression of IA-2 (either PTP or a
longer central region construct) and optimize expression and purification of the 96/3 scFv in order to prevent future losses to degradation.

Knowledge of the composition of conformational epitopes enables the design of both peptides representing epitopes, and monoclonal antibodies. Both peptides and antibodies could be of use in diagnosis and treatment of type 1 diabetes, and other diseases (Routsias, Vlachoyiannopoulos et al. 2006; Gomara and Haro 2007). Knowledge of peptides and antibodies representing immunodominant epitopes in type 1 diabetes could lead to the design of a vaccine in the future, as it has for many infectious diseases (Hans, Young et al. 2006).
Chapter 6  Mapping of antibody epitopes within the Juxtamembrane domain of IA-2.

6.1 Introduction:
Development of Type 1 diabetes is closely associated with expression of islet-antigen reactive autoantibodies. However, the method of antibody recognition of particular epitopes within these antigens remains unclear. As a major target of the autoimmune response in diabetes, IA-2 autoantibodies are predictive of future disease development (Christie, Genovese et al. 1994). Autoantibodies to the juxtamembrane (JM) domain of the IA-2 molecule are known to appear before reactivity to the PTP domain of the molecule can be detected, and according to results of a study in Finland, both the presence of IA-2 JM autoantibodies, and levels of reactivity are very strongly associated with pre diabetes (Hoppu, Harkonen et al. 2004).

The frequency of IA-2 autoantibody positivity varies both with age, and HLA type, including reported associations with HLA-DR4, DR7 and DR9 (Williams, Aitken et al. 2008; McLaughlin, Gulati et al. 2014). Therefore, this study used selected single amino acid mutations to identify relationships between specific HLA alleles and antibody reactivity to the JM domain epitopes identified. IA-2 autoantibodies are most common in individuals expressing HLA-DR4/DQ8 genotype (Gorus, Goubert et al. 1997; Hawa, Rowe et al. 1997). The expression of this high risk HLA class 2 is associated with both development of antibodies to IA-2 and approximately 50% of genetic susceptibility to the disease (Nerup, Platz et al. 1974; Lambert, Gillespie et al. 2004). A study by Bearzatto et al (Bearzatto, Naserke et al. 2002) revealed lower occurrence of antibodies to the JM1 epitope (within the 601–620 amino acids sequence of the molecule) in HLA-DR3/4, DR1/4 or DR4/13 subjects. These results suggested that a more in depth study of the link between HLA and recognition of JM domain epitopes needed to be undertaken. Therefore, the effects of several major type 1 diabetes susceptibility HLA alleles on antibody responses to IA-2 JM domain epitopes were examined in this study.

Fine mapping of the epitopes in the IA-2 JM domain, and genetic predisposition towards autoimmunity to these epitopes, could clarify our understanding of the initiation and progression of autoimmunity in Type 1 diabetes.

6.2 Aim
This research group has previously used 20mer linear synthetic peptides to coarse map epitopes within the JM domain. Information was also obtained for the role of several specific amino acids. The purpose of this section of the project was to extend this knowledge, by looking further into the
regions defined using peptide inhibition, and extending the fine mapping by site directed mutagenesis to cover the whole region defined by peptide inhibition. This was achieved using:

- Inhibition of serum antibody binding using 20mer linear peptides representing sections of the juxtamembrane domain.
- Site directed mutagenesis of amino acids within the region defined using peptide inhibition
- Analysis of patterns of antibody binding
- Analysis of associations between patterns of antibody binding and HLA type.

6.3 Methods:

6.3.1 Generation of a chimeric JM-PTP1B construct for radioligand binding assays (RBA):

Previous studies on antibody reactivity to the JM domain were performed using a truncated section of IA-2, representing amino acids 605-693 (Hatfield, Hawkes et al. 1997). Unfortunately, radiolabeling of this construct with $^{35}$S methionine was inefficient, as it contains only two methionines. In order to improve recovery of radiolabel in our assays, we developed a chimeric construct: joining the JM domain of IA-2 with the PTP domain of PTP1B, which has high homology with the PTP domain of IA-2, but does not bind antibodies in type 1 diabetes.

The original JM construct was amplified using a forward primer containing an EcoRI site (GAATTTC) and a reverse primer which overlaps into the sequence of PTP1B. In a separate reaction, a PTP1B construct generated in this group was amplified using a forward primer overlapping into the JM sequence of IA-2, and a reverse primer containing a Sall restriction site (GTCGAC). PCR reactions were then gel purified, and overlapped, using by combining PCR products at equal concentrations, in the presence of polymerase and dNTPs, and running 10 cycles of 94°C-1 minute, 50°C, 25 minutes and 72°C for 1 minute.
This product was then reamplified using the forward primer from the JM reaction, and the reverse primer from the PTP1B reaction, for ligation into the pTNT vector (Promega), shown in Figure 6-1, a vector designed specifically for transcription/translation using the pTNT system, used to generate radiolabeled protein.

6.3.2 **Characteristics of patients used in this study:**

Patients were recruited from clinics in West Yorkshire and King’s College Hospital, London, UK with informed consent and approval from the Yorkshire and the Humber, Bradford, Leeds and the King’s College Hospital Research Ethics Committees for studies on the specificity of B cell and T cell responses in disease. The conditions of ethical approval for the study in Yorkshire restricted recruitment to patients ≥12 y of age, leading to an under-representation of young children, although all patients were within six months of diagnosis of type 1 diabetes. Type 1 diabetes was diagnosed according to World Health Organization criteria. 140 patient samples were suitable for this study. The mean age was 18.8 years, and 94/140 (67%) were male. Blood samples were taken, and analyzed for antibody reactivity and HLA typed. Of a total of 136 patients, 50 recognized epitopes within the JM domain. One of these had an insufficient volume of serum for full characterization of autoantibody epitopes, leaving 49 for the JM epitope analysis. Of the 49, 63% were male, and the average age of all patients in this study was 19.9 years old. 30 normal controls were also recruited, with a mean age of 21.9, 46% males.

6.3.3 **Antibody analysis**

Antibodies to glutamate decarboxylase (GADA), zinc transporter-8 (ZnT8A), the cytoplasmic (IA-2ic, residues 606-979) and PTP (694-979) domains of IA-2 were analyzed by radioligand binding assay as previously described (Lampasona, Bearzatto et al. 1996; Christie, Roll et al. 1997; Hatfield, Hawkes et
al. 1997; Wenzlau, Juhl et al. 2007). The sensitivity and specificity of the IA-2 antibody test in the 2012 Immunology of Diabetes Society Autoantibody workshop was 62% and 100%, respectively. All patients recruited during the course of this study were analyzed for antibodies to the major islet cell antigens, excluding insulin autoantibodies as all were treated with insulin. Patients categorized as positive for IA-2A were then retested for sub-specificities of IA-2A, using three constructs to determine epitope reactivity within the IA-2 molecule.
6.3.4 **Constructs for epitope determination:**

The constructs used in this study are shown in Figure 6-2. The IA-2 IC construct was used to define all patients reactive to the intracellular region of IA-2. This was generated using the pSP64 poly (A) (IA-2 605–979), as was the central region (643-937) construct. The PTP domain construct was inserted into the pGEM-T-easy vector (with the insert representing amino acids 693–979). Finally, the JM-PTP1B (IA-2 605-693) chimera was generated and ligated into the pTNT vector shown in Figure 6-1 (Promega, Madison, WI) under the control of the SP6 promoter (Lampasona, Bearzatto et al. 1996).

6.3.5 **Site directed mutagenesis of IA-2 JM construct:**

Insertion of single nucleotide changes into the JM domain of the JM-chimeric construct cDNA using the Quick Change (Stratagene) method. PAGE purified, mutagenesis-specific primer pairs were synthesized for each target amino acid. The following amino acid residues were individually changed to alanine by mutagenesis of the relevant codon: K609, E610, R611, L612, L615, G616, E618, G619, H621, G622, D623, T624, T625, F626, E627, Y628, Q629, D630, L631, C632, R633, Q634, H635, M636, T638 and K639.

6.3.6 **In vitro translation and immunoassay:**

Plasmid DNA was purified using the Bio-Rad Quantum Prep plasmid purification system (Bio-Rad, Berkeley, California, USA) as described in Table 2-6. DNA was then transcribed and translated in vitro using a TnT SP6-Quick-coupled transcription translation system (Promega) in the presence of 35S methionine according to the manufacturer’s instructions.

In the RBA, 5μl of serum was added to 20μl of IMP buffer (10mM HEPES, 150mM NaCl (pH 7.4), 10mM benzamidine, and 2% Triton X-100, 0.5 %BSA and 0.5mM methionine) incorporating 20,000 cpm of labeled translate proteins in a 96-deep well plate (Starlab) and incubated overnight at 4°C. Antibodies complexed with labeled antigen were recovered by adding 50% protein A Sepharose in 10μl of IMP buffer. The assay was then incubated for 1 h at 4°C with shaking. The assay was then transferred to a filter plate (NUNC, Denmark), and washed through five times with 200μl of IMP buffer, followed by two washes with water. Plates were then dried for 1 hour at 37°C, and precipitated labeled protein was resuspended in 100μl of Microscint 50 (Packard). The complexes were then counted for 30 seconds in a Chameleon (Hidex) scintillation counter for measurement of the recovered cpm.

Positivity for major islet autoantigens was measured relative to a standard curve prepared by measurement in each assay of a serum with known autoantibody levels, serially diluted in normal serum (AB+).
6.3.7 **Peptide blocking experiments and amino acid substitutions:**

Regions of IA-2 recognized by patients were studied using addition of synthetic 20mer peptides, using a modified RBA, with the addition of an excess of one of three 20mer synthetic peptides. The peptides used were:

1. Amino acids 601-620
2. Amino acids 621-640
3. Amino acids 611-630

Competition was conducted by incubating the sera with the IA-2JM domain in the absence or presence of ~5μg of synthetic IA-2 JM domain peptide, with radiolabeled IA-2JM or IA-2 IC and performing the assay as described above. Sera were considered to recognize a JM epitope represented within the peptide if they were inhibited (>25%) by peptide. Serial dilutions of high antibody level sera, to sub-maximal binding, were performed on high antibody level sera to ensure inhibition was not masked by high levels of binding. Dilutions were made in normal human serum. Similar dilutions were performed to verify inhibition of alanine substitutions. Inhibition of binding was calculated as a percentage of wild type binding.

6.3.8 **HLA typing:**

Genomic DNA was extracted from white blood cells contaminating red blood cell pellets from plasma and PBMC separation. Genomic DNA was precipitated using ethanol, and cleaned up using a DNA clean and concentrator kit (Zymo, CA). HLA types were determined by PCR gel analysis, using specific primer sets for HLA-DR and HLA DQ using primers described by Bunce et al (Bunce and Passey 2013). All HLA typing was performed by Dr Kerry McLaughlin. HLA DP was not tested and HLA-DR4 patients were not subtyped.

6.3.9 **Statistical analysis**

Relationships between inhibitory effects of individual amino acid substitutions and binding of antibodies in Type 1 diabetic patients to mutated IA-2 JM constructs was analyzed by hierarchical cluster analysis using the furthest neighbor method. Cluster analysis was performed using the Statistics Package for Social Sciences version 22 (SPSS; IBM, Portsmouth, UK). The significance of differences between groups in inhibitory effects of individual or clusters of amino acid substitutions were analyzed by two-way analysis of variance (ANOVA) using Sidak’s correction for multiple comparisons. ANOVA was performed using Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA).
6.4 Results:

6.4.1 Selection of patient samples

Antibody specificity of patient serum samples were tested using radioligand binding assays for three of the major islet autoantigens, IA-2ic, GAD65, and ZnT8. Assay performance was verified by participation in the IASP antibody workshop (2012). All samples that tested positive for IA-2A were subsequently retested for reactivity to specific IA-2 constructs, using three constructs to represent the JM domain (amino acids 605-693), the PTP domain (694-979), and the central region of the IA-2 cytoplasmic domain (643-937) shown in Figure 6-2.

![Table showing antibody reactivity by radioligand binding assay](image)

<table>
<thead>
<tr>
<th>Antibody reactivity by radioligand binding assay</th>
<th>Number (%) Type 1 diabetic patients positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA-2 intracellular region 605-979</td>
<td>IA-2 ic: 94 (69%)</td>
</tr>
<tr>
<td>JM Central C terminus 605-693</td>
<td>IA-2 JM 50 (37%)</td>
</tr>
<tr>
<td>JM PTP1B PTP domain 605-979</td>
<td>IA-2 PTP.77 (57%)</td>
</tr>
<tr>
<td>IA-2 PTP domain 643-637</td>
<td>IA-2 CR: 60 (44%)</td>
</tr>
</tbody>
</table>

Figure 6-2: Showing antibody reactivity in patients from London and Yorkshire, recruited within 6 months of diagnosis of diabetes. Reactivity to the PTP domain is most common, with JM reactivity found in 37% of IA-2 reactive patients. 69% of all patients recruited were IA-2 reactive.

As seen in Figure 6-2, 69% of patients recognized the whole IA-2 molecule, 37% of IA-2 reactive patients recognized the JM domain, 57% recognized the PTP domain and 44% recognized the central region.

6.4.2 Generating a chimeric JM construct:

The JM domain of IA-2 was initially amplified by PCR using a full length IA-2 construct as a template. This was then used to generate a chimeric construct of the IA-2 JM domain linked to the PTP domain of PTP1B using an overlap PCR reaction (Figure 6-3).
Figure 6-3: JM-PTP1B chimera, created using overlap PCR. The gel shows JM product for overlap, PTP1B product for overlap, and the final overlapped product, with some residual JM alone and PTP1B alone.

The PCR product representing the full length JM-PTP1B chimera was digested alongside the pTNT vector, using EcoRI and SalI, as shown in Figure 6-4, and ligated into the digested pTNT vector to generate a construct suitable for in vitro transcription and translation of the radiolabeled JM chimera.

Figure 6-4: shows EcoRI/SalI digest of pTNT alone, and pTNT with ligated JM-PTP1B chimera.

Comparisons of radioactivity incorporated into the JM-chimera, compared with the shorter JM construct without a PTP domain showed an average ten-fold increase in cpm recovered, from ~10 000 cpm/µl to ~100 000 cpm/µl.

Ten type 1 diabetic patient sera were tested against the PTP1B construct alone, and PTP1B-JM chimera in order to confirm that there was no reactivity to the PTP1B section of the construct. Results shown below in Figure 6-5 confirm the lack of reactivity to the PTP1B construct, whilst several patients known to recognize the short JM construct were able to recognize the JM chimera.
Figure 6-5: To confirm no patients exhibited reactivity to the PTP1B section of the JM chimera, ten diabetics were tested against both the PTP1B section of the chimera, and the JM chimera. None of the diabetic patients showed any reactivity to the PTP1B construct alone.

In order to further validate the chimeric JM-PTP1B construct as a tool for detecting antibodies to the JM region, 57 patients, known to recognize the short JM construct, collected from both London and Yorkshire were tested alongside 24 healthy controls. The controls were used to determine a threshold for JM positivity, and the diabetic patient samples were used to verify the positive response to the antigen. Samples were tested alongside a rabbit polyclonal antibody, reactive to the JM domain of IA-2, and sample reactivity was plotted as a percentage of the total binding of the polyclonal antibody (JM antibody units), shown in Figure 6-6. The cut-off was defined as the mean +3 standard deviations of antibody units in the control samples, which was 0.43 units. All 57 samples known to recognize the short construct were also positive for antibodies to the chimera.
Figure 6-6: Assessing specific binding of the JM-chimera, and setting a threshold to define JM positive samples, using a full panel of 140 recent onset diabetic patients, and 30 age matched controls.

6.4.3 **Defining JM domain epitopes recognized by Type 1 diabetic patients:**

A further 140 patients were subsequently tested against the chimeric construct, to identify a panel of JM antibody positive samples for testing JM reactivity. In order to further define reactivity within the JM domain, 50 JM reactive patient samples were positive for antibodies to the JM chimera construct; of these 49 were then used for subsequent studies. All 49 patients were then tested for reactivity to particular JM regions, using 20mer peptides to represent the major JM antibody epitopes.

As shown in Figure 6-7, whilst 97% of patients are inhibited by peptide 621-640, and a similar proportion are inhibited by peptide 611-630, only 49% of patients recognize the epitope represented by peptide 601-620. Patients inhibited by the 601-620 peptide, shown by red spots, consistently show higher levels of inhibition than those who are unaffected, shown by purple spots. Antibody binding for almost all samples was inhibited by the 611-630 and 621-640 peptides as seen in Figure 6-7, suggesting that the 621-640 region plays an important role in binding of all JM autoantibodies. Inhibition by 611-630 and 621-640 peptides was unaffected by JM antibody levels. Patients were subdivided, using this data, into those inhibited by peptide 601-620, or not inhibited by addition of this peptide.

Circled points, in peptide 621-640 and peptide 611-630 assays show samples that were only inhibited by one peptide, suggesting that they bind more restricted epitopes than the majority of sera. There were three patients inhibited only by the 621-640 peptide, and two patients only inhibited by 611-630 peptide in this study sample.
Figure 6-7: Showing inhibition of binding of IA-2 JM construct using linear 20mer peptides. Addition of peptides at high concentrations to radioligand binding assays inhibit binding of native JM constructs in patients who recognise epitopes defined by a particular peptide. Three peptides were used: 601-620, 611-630 and 621-640. Patient recognition of epitopes can be divided into two groups: those that recognise all three epitopes (red spots) and those that do not recognise the JM1 epitope (purple spots). Nearly all patients are inhibited by 611-630 and 621-640.

6.4.4 Effect of antibody levels on peptide inhibition:
Analysis of inhibition by peptides showed that serum samples with high levels of antibody tended not to be inhibited by addition of 5µg of the 20mer peptide 601-620, as seen in Figure 6-8A. Both peptide 621-640 and peptide 611-630 showed inhibition of sera with high levels of antibody (Figure 6-8B, C).
Figure 6-8: Comparison of inhibition of antibody binding by addition of an excess of peptide representing amino acids 601-620 (A.), 611-630 (B.) or 621-640 (C.) with antibody levels. Inhibition by the 601-620 peptide is associated with lower antibody levels.

The results above raised the possibility that lack of inhibition by peptide 601-620 in some patients was the consequence of the inability of the peptide to compete with very high levels of antibody to that specific epitope. To determine whether this may be the case, the experiment was repeated after dilution of the serum samples to levels where there was submaximal binding to the radiolabeled antigen. Lack of peptide inhibition remained after dilution of 1:4. Average peptide inhibition at neat, 1:2 and 1:4 was 1.26% and 0.26%, as can been seen Figure 6-9, where no significant difference can be seen between neat and 1 in 4 dilutions.
6.4.5 **Fine mapping of epitopes using site directed mutagenesis of amino acids:**
Inhibition of binding using peptide blocking provides a crude estimate of epitope specificity. Single amino acid substitution can provide a clearer picture of which amino acids are important for binding to specific epitopes.

To further clarify patient reactivity to JM epitopes, sequential substitution of amino acids in the JM domain was performed using alanine scanning mutagenesis. Amino acids from 608-639 were substituted with alanine. Inhibition of binding was measured in comparison to binding to the wild type JM chimera construct.
Figure 6-10: Individual sera reactive to specific epitopes. 49% of patient sera are inhibited by the 601-620 peptide (19/49). All 601-620 sera are also inhibited by peptides 611-630 and 621-640. Nearly all sera (98%) are reactive to the region between 611-640. Two sera were reactive to the 611-630 region only. Three sera were reactive to the 621-640 region only.

Individual sera characterized by peptide blocking have a heterogeneous response to single amino acid substitution. However, clear areas of similarity remain between sera. As seen in Figure 6-10A 601-620 reactive sera are all affected to some degree by substitution of amino acids from 611-619. The common amino acids from 634-636 also affect binding of individual 601-620 reactive sera.
Figure 6-10B shows the inhibition pattern of sera inhibited only by the 621-640 peptide, which are affected by substitution of 609, the key 624-627 amino acids, and the common 634-638 region, but to a lesser degree by substitution of amino acids in the 601-620 area. Individual sera inhibited by addition of both 611-630 and 621-640 peptides show the importance of both the amino acids 625-627 and 609, and the 631-639 common area (Figure 6-10C).

The sera inhibited by only the 611-630 peptide have very few consensus amino acids: 615, 616, 621, 624, 627, 628, 631, 633 and 636 (Figure 6-10D). Unfortunately, with such low numbers recognizing single epitopes, understanding the pattern of amino acids contributing to this epitope is difficult.

Inhibition by substitution of amino acids 615, 631, 633, 634, 635 and 636 influenced antibody binding of samples irrespective of peptide inhibition observed in competition binding studies.

Inhibition by individual amino acids was clearly different between samples, dependent on inhibition by the 601-620 peptide. Patients were therefore subdivided according to inhibition of JM antibodies by the 601-620 peptide.

Samples in which high levels of inhibition were seen by substitution of amino acids 611 or 612 had much higher mean inhibition by addition of the 601-620 peptide. Samples in which antibody binding was inhibited by the 601-620 peptide were also likely to be inhibited by alanine substitutions of amino acids 616, 618 and 619. (Figure 6-10).

The inhibition of binding of JM-chimera by alanine substitution of sequential amino acids in all patients inhibited by the 601-620 peptide is shown in Figure 6-10A. Amino acids within the region 611-619 showed strong inhibition in this group of patients. Figure 6-10D shows 611-640 reactive patients require amino acids 609, 621, 622, 625, 626 and 627 for binding. Both 601-620 inhibited samples and 611-640 inhibited samples are affected by substitution of amino acids between 633-636.

To clarify the relationships between classification of sera by peptide blocking and inhibition by single amino acid substitution, relationships between amino acids was analyzed using a hierarchical cluster analysis, and a dendrogram was created, using scaled Euclidian distances, and calculating the distance to the furthest neighbor. The dendrogram, shown in Figure 6-11B, allows the creation of clusters of amino acids, forming the basis of an epitope, of common amino acids required for binding of all sera or of those that have little or no effect on binding.
Figure 6-11: A. Influence of substitutions of individual amino acids within the 608-639 region of IA-2 expressed in the IA-2 JM domain construct on binding of antibodies in sera from 49 JM antibody positive patients grouped according to inhibitory effects (white bars not inhibited, black bars inhibited) of peptide 601-620. The significance of differences of effects of individual amino acid substitutions between groups affected or not affected by the 601-620 peptide is shown: * p<0.05, ** p<0.01, *** p<0.001. 

B. Dendrogram illustrating results of cluster analysis of data from experiments shown in panel B evaluating similarities in inhibitory effects of individual amino acid substitutions. Residues with similar inhibitory effects are grouped into clusters defined by distance in the dendrogram being ≤10.
Correlation between inhibition by specific amino acid substitution and peptide inhibition gives a clearer idea of relationships between particular amino acids, and the role they may play in epitope formation. The relationships between amino acids can be clearly defined using a dendrogram, which creates a tree diagram of relationships between inhibitory effects of individual amino acids: The analysis identifies 6 distinct clusters grouped according to distance in the dendrogram being ≤10.

Those samples categorized according to inhibitory effects of the 601-620 peptide showed different mean inhibitory effects of substitutions of amino acids within each cluster. This demonstrates the difference in epitopes recognized, as shown in Figure 6-11B, with amino acids 609, 625, 626 and 627 forming the basis of the 621-640 epitope. The amino acids form cluster 6 in Figure 6-11B. Figure 6-12 shows association of clusters with JM1 peptide inhibition. Cluster 3 associates positively with inhibition by P1, whilst Clusters 5 and 6 associate negatively.

Substitutions of amino acids in cluster 4 (615, 616, 631, 633, 635, 636) affected most samples irrespective of peptide inhibition, whereas those in cluster 1 (630 and 639) and cluster 2 (608, 610, 624, 628, 629, 632, 638) rarely affected antibody binding.

Amino acids 611,612, 618,619 and 623 form the basis of the 601-620 epitope (see above, cluster 3, Figure 6-11B) and the inhibition by substitution of these amino acids correlates positively with inhibition by 601-620 peptide, whilst those in cluster 5 (621, 622) and cluster 6 (609, 625, 626, 627) showed strong inhibitory effects in those unaffected by the peptide.
6.4.6  **Effect of antibody levels on single amino acid substitutions**

Inhibition by individual alanine substitutions was mostly independent of JM antibody levels in individual sera, with effects of antibody levels only seen in some residues; 611 612 and 619, where minimal inhibition was observed in patients with high levels of JM antibodies (Figure 6-13).

![Diagram showing inhibition by alanine substitution](image)

**Figure 6-13:** Association between antibody levels of samples, defined by comparison of levels with a rabbit polyclonal antibody to the JM domain (Rabbit polyclonal binding = 100 antibody units) and inhibition by alanine substitution of individual amino acids. Only binding of amino acids 611 and 612 are significantly affected by antibody levels.

Serial dilution of high antibody level samples showed no significant increase in inhibition by amino acid substitution, as shown in Figure 6-14, where no increase in inhibition can be seen when sera are diluted 1:4.
6.4.7 **Defining links between JM antibody binding and HLA-DR4**

Samples from 140 Type 1 diabetic patients obtained within 6 months of disease diagnosis collected from Yorkshire and London were genotyped for HLA-DRB1 and HLA-DQB1 alleles and analyzed for antibody reactivity to the cytoplasmic domain of IA-2 (IA-2ic) and to constructs representing the PTP domain (residues 694-979) as well as the chimeric JM construct (606-700) domain of IA-2. Of these 69% were positive for cytoplasmic domain antibodies, 56% positive for antibodies to the PTP domain construct and 35% for JM antibodies.

HLA typing of these samples, performed by Dr. Kerry McLaughlin, showed DR4 positive patients, were more likely to recognize the whole IA-2 molecule, the central region of IA-2, and the JM domain, as seen in Table 6.1. There is a distinct association between the presence of HLA-DR4 with antibodies to the whole JM domain, as seen in Table 6.1, showing the total number of patients representing each HLA type capable of recognizing one or more epitope regions of IA-2. The majority expressed HLA-DR4 (77%) and 59% expressed both DR4 and DQ8 alleles. The high risk HLA DR3/4 DQ2/8 was expressed in 15 of the 49 patients in this group (30%).
Table 6-1: Characteristics of IA-2A positive patients recruited from London and Yorkshire. Showing total number of individuals of each HLA type capable of recognizing each epitope region of the IA-2 molecule, and percentage of positive patients in brackets.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>IA-2ic-Ab+</th>
<th>IA-2-PTP-Ab+</th>
<th>IA-2-JM-Ab+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL</td>
<td>140</td>
<td>96 (69%)</td>
<td>78 (56%)</td>
<td>51 (36%)</td>
</tr>
<tr>
<td>HLA-DR4</td>
<td>84</td>
<td>65 (77%) **</td>
<td>53 (63%)*</td>
<td>39 (46%) **</td>
</tr>
<tr>
<td>HLA-DQ2</td>
<td>88</td>
<td>59 (67%)</td>
<td>33 (53%)</td>
<td>31 (35%)</td>
</tr>
<tr>
<td>HLA-DQ7</td>
<td>25</td>
<td>17 (68%)</td>
<td>15 (60%)</td>
<td>11 (44%)</td>
</tr>
<tr>
<td>HLA-DQ8</td>
<td>63</td>
<td>52 (83%) **</td>
<td>41 (65%)</td>
<td>31 (49%) **</td>
</tr>
<tr>
<td>HLA-DR3/4</td>
<td>37</td>
<td>30 (81%)</td>
<td>22 (59%)</td>
<td>18 (49%)</td>
</tr>
<tr>
<td>HLA-DR4/4</td>
<td>12</td>
<td>8 (67%)</td>
<td>7 (58%)</td>
<td>7 (58%)</td>
</tr>
<tr>
<td>HLA-DR3/non-DR4</td>
<td>35</td>
<td>18 (51%)*</td>
<td>18 (51%)</td>
<td>6 (17%) **</td>
</tr>
<tr>
<td>HLA-DR7/non-DR4</td>
<td>16</td>
<td>12 (74%)</td>
<td>9 (56%)</td>
<td>7 (44%)</td>
</tr>
</tbody>
</table>

6.4.8 The effect of high risk HLA types on recognition of specific JM epitopes defined by the cluster analysis

The definition of key amino acids within the two JM epitopes is useful for determining any underlying links between individual HLA type and ability to recognize specific IA-2 epitopes. Clustering of small groups of amino acids allows the definition of regions clearly affected by HLA type. Consistent with previous studies in
Table 6-1, there is a link between expression of HLA-DR4 and recognition of epitopes within the JM region. The majority of IA-2-JM antibody positive patients in the epitope analysis expressed HLA-DR4 (n=37) or HLA-DR7 (n=7). Patients expressing HLA-DR3/4, DR1/4 or DR4/13 showed significantly lower mean inhibition by substitutions of cluster 3 residues (8.4±4.7%, n=26) than those with other HLA genotypes (30.9±5.4%, n=23; p<0.005).

Figure 6-15 demonstrates that patients expressing HLA-DR7 had significantly higher mean inhibition by substitution of cluster 3 amino acids (611, 612, 618, 619, and 623) than those with HLA-DR4.

Figure 6-15 shows the association of expression of DR7 with cluster 3 (JM1- amino acids 611,612,618,619,623). There is a positive association between expression of HLA-DR7 (not HLA-DR4) with inhibition by substitution of these amino acids, suggesting that HLA-DR7 predisposes individuals to recognize amino acids in this epitope region, in contrast to antibodies from patients expressing HLA-DR4.

![Figure 6-15](image)

Figure 6-15: Expression of HLA-DR4 has a negative effect on recognition of Cluster 3 (611,612,618,619, 623), making patients less likely to recognize JM1 epitopes. P<0.005, 2 way ANOVA with Sidak’s correction for multiple comparisons.

Within HLA-DR4 patients, significant differences in mean inhibition by residues 621 and 622 were observed between those with HLA-DQ8 and -DQ7 alleles. The results demonstrate influences of both HLA-DR and DQ genes on autoimmune recognition of the IA-2 JM domain. Individuals expressing both HLA-DR4 and DQ8 alleles are more likely to require amino acids 621 and 622 (cluster 5) for binding to the JM region than patients expressing HLA-DQ7. Of these, the clearest association is with G622. Figure 6-16 shows a clear increase in inhibition by substitution of amino acid G622, and the presence of HLA DQ8 in DR4 positive individuals. G622 may form a part of the JM2 epitope previously reported, implying it is necessary for binding of JM2 epitopes in many DR4/DQ8 patients, and is negatively associated with recognition of the JM1 region.
The results define, in part both the 601-620 and 611-640 epitopes previously described by Lampasona et al and Bearzatto et al (Lampasona, Bearzatto et al. 1996; Bearzatto, Naserke et al. 2002; Bearzatto, Lampasona et al. 2003; Lampasona, Belloni et al. 2008). They also demonstrate a clear role for HLA DQ8 in epitope recognition by patients.

HLA DQ8 expression promotes recognition of cluster 5, amino acids 621 and 622. The effect is independent of the association with HLA-DR4 for antibodies to JM regions previously defined. Amino acids 621 and 622 lie within the previously defined 621-640 epitope, however, inhibition by substitution of these amino acids does not correlate with inhibition by addition of peptide, suggesting the presence of an alternative epitope.

6.5 Discussion:

Reactivity to the JM domain appears early in disease onset (Naserke, Ziegler et al. 1998), and the appearance and/or persistence of autoimmunity to this region is associated with HLA-DR4 (McLaughlin, Gulati et al. 2014) (Bearzatto, Lampasona et al. 2003), suggesting that the immune response to epitopes within this region may be determined by this allele. Responses to epitopes within the JM domain have been shown to be heterogeneous (Lampasona, Belloni et al. 2008), therefore it is possible that autoimmune responses to individual JM domain epitopes are associated with different HLA genotypes (Bearzatto, Naserke et al. 2002). Data for the HLA associations shows that HLA type does not predispose towards classification of epitope recognition of epitope regions broadly defined by peptide inhibition (601-620, 611-640) but does show a predisposition of certain HLA types to recognize particular amino acid clusters, which may not be associated with a particular epitope definition. This suggests that there are in fact multiple epitopes within the JM domain, and the immune response to this area is more complex than previously thought.
The data presented in this section of the study are consistent with the presence of two broad regions within the JM domain of the IA-2 molecule contained within amino acids 609-621 (JM1) and 619-631 (JM2), as previously described by Bearzatto et al (Bearzatto, Naserke et al. 2002) using chimeric IA2/IA2β molecules, representing short stretches of the JM domain. The same group later defined a third epitope in this region, using IA-2 JM/Trx fusion protein (Lampasona, Belloni et al. 2008). The regions described in the Lampasona and Bearzatto studies (Bearzatto, Naserke et al. 2002; Lampasona, Belloni et al. 2008) cover amino acids 609-631 and describe three epitopes, JM1, 2 and 3. The approach used in this study was to inhibit binding using synthetic peptides representing different but overlapping areas of the molecule: 601-620 (similar to JM1) 621-640 (similar to JM2) and 611-630 (similar to JM3).

Inhibition of binding of antibodies in patient sera using peptides reveal two major binding patterns: patients with immunoreactivity blocked by addition of the 601-620 peptide, and patients with antibody binding not inhibited by addition of this peptide. These results suggested the presence of two major epitope areas, which were further studied using alanine scanning site directed mutagenesis.

Fine mapping of epitopes within the regions defined by peptide blocking using site directed mutagenesis showed better definition of specific epitopes within these regions. In order to perform this study a chimeric molecule incorporating the PTP domain of PTP1B (694-979), which is not recognized by antibodies in diabetes, linked to the JM domain of IA-2 amino acids 606-700 was generated. This allowed more efficient incorporation of radiolabeled methionines during transcription translation, and lower background in radiobinding assays. Sequential site directed mutagenesis of suitable amino acids in this molecule mapped several different regions critical for patient antibody binding. As predicted, using a PTP1B backbone for the JM region allows exposure of the antibody epitopes of the protein in a conformation which is recognizable to patient serum antibodies. In the study using Trx as a backbone (Lampasona, Belloni et al. 2008) some patients produce antibodies to the backbone protein, preventing study of those patients. Many patient sera react to IA-2β, limiting the use of this protein as a backbone for the JM domain. In our studies, no patients reacted with PTP1B, suggesting this is an ideal backbone protein for the short construct representing the JM.

Amino acids were subdivided into clusters, dependent on inhibitory effects: this generated six separate clusters of amino acids, which could be formed into four groups:
- Clusters 1 and 2 in which alanine substitution had minimal effects on antibody binding for the majority of sera
- Cluster 3 amino acids, which affected binding 601-620 peptide inhibited patients.
- Clusters 5 and 6 which only affected patients with no reactivity to the 601-620 peptide
- Cluster 4 residues, which were capable of inhibiting binding of antibodies in most sera, irrespective of effects of the 601-620 peptide.

The JM1 (Bearzatto, Naserke et al. 2002) epitope requires amino acids 611, 612, 618 and 619. This region is clearly represented in patient sera blocked by addition of peptide representing the 601-621 region of the antigen. The region representing the JM2 epitope is broader, with amino acids 609, 625,626 and 627 contributing to its formation. At least one of the amino acids 615, 631, 633-636 are important for binding of all classes of JM antibodies. Lampasona et al (Lampasona, Belloni et al. 2008) showed heterogeneity in individual amino acids contributing to serum recognition of defined JM epitopes, when using site directed mutagenesis to target amino acids 619, 621, 622, 623,624, 625, 626, 627, 628, 629, 630 and 631. Using a more extensive panel of patients, a longer construct representing a larger area of the JM region, and more extensive mutagenesis, this study confirms the heterogeneity of the antibody response to the JM region, whilst showing some common features both within epitopes and between epitopes. The data generated shows that contrary to previous assumptions, the amino acids which contribute to epitope formation in the JM domain are clustered, rather than linear. In some cases, amino acids contributing to epitope formation are non-sequential, with amino acid 608 forming a cluster with 625, 626 and 627 (cluster 6). These results suggest that substitution of this residue may either disrupt conformation of the entire 625-627 region, possibly disrupting ionic interactions, or residue 609 is folded into close proximity with the 625-627 region and participates directly in antibody binding.

Antibody recognition of the JM domain as a whole is already known to be associated with expression of HLA-DR4. The definition of individual epitopes by clustering of amino acids further reveals influences of different HLA alleles on antibody recognition of the JM domain. Results from this study are supported by findings of the Bearzatto group, individuals expressing HLA-DR3/4 DR1/4 or DR4/13 were significantly less likely to be inhibited by substitution of amino acids in cluster 3 than were subjects with other HLA genotypes. Expression of the HLA-DQ8 allele in patients also expressing HLA-DR4 was associated with inhibition by cluster 5 amino acids, implying that this allele may also influence the specificity of the IA-2 JM immune response. This association may be directly related to the association of DQ8-restricted T-cell responses to T-cell determinants, which could
include peptides within the region 601-633 that have been shown to specifically stimulate T-cell responses in HLA-DQ8 transgenic mice (Chang and Unanue 2009).

Data generated in this study also suggested a possible role for HLA-DR7; inhibition of JM domain binding by alanine substitution of amino acids within the cluster 3 (JM1) epitope was more common in patients with HLA-DR7 than those with HLA-DR4. HLA-DR7 has been shown to have either protective or neutral influences on disease development, depending on the associated DQA1 allele expressed (Noble, Johnson et al. 2011). Positive association of HLA-DR7 and antibodies to the whole cytoplasmic domain of IA-2 has been previously reported, however in that study, a negative association was found with JM antibodies (Williams, Aitken et al. 2008); both studies do show an influence of HLA-DR7 on disease development, and the Williams study uses a chimeric IA-2JM molecule, missing amino acids 633-636, which as shown in this chapter, play a key role in optimal binding of the JM domain. Samples used in this study were not genotyped for HLA-DQA1; therefore any effects of the DQA1 allele could not be clarified. The results of this study do suggest that there is an effect of either DR7 or the linked DQ on antibody binding of JM (Bearzatto, Naserke et al. 2002).

This study further defines autoantibody epitopes within the JM domain, which is known to be recognized in the early stages of islet autoimmunity. The results underline the importance of the role of HLA-DR and DQ gene products on the specificity of the autoantibody response in type 1 diabetes, and pave the way for a greater understanding of the T cell determinants associated with the B cells response to JM epitopes.
Chapter 7  Relationships between juxtamembrane and PTP domain epitopes on the IA-2 autoantigen

7.1  Introduction

Diversification or spreading of the immune response within and between specific autoantigens is an important factor in progression from first appearance of autoimmunity to clinical onset of Type 1 diabetes (Brooks-Worrell, Gersuk et al. 2001). Whilst the early phase of the autoimmune process appears to be focused on the JM domain of IA-2 as the disease progresses, reactivity to the PTP domain, and to the sister protein tyrosine phosphatase, IA-2β, appears. Recent studies have demonstrated that T cell responses to a peptide (841-861) representing a part of the central region of the IA-2 molecule are associated with antibodies to the JM domain of the protein (McLaughlin, Richardson et al. 2015), suggesting that there may be some interaction of immune responses between these two domains. The mechanism underlying this association was proposed to involve enhanced processing and presentation of PTP region peptides by JM-reactive B cells to T cells upon antigen presentation.

Stabilization of PTP domain T cell epitopes by antibodies to the JM domain may be the consequence of structural alignment of the JM B cell epitope with the central region peptide (841-860), the latter of which is contained within another major autoantibody epitope (827-862) (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009). Whilst a crystal structure of the PTP domain of the IA-2 molecule was published in 2007 by Kim et al (Kim, Jeong et al. 2007), very little is known about the structure of the JM domain and its relationship with the PTP region of IA-2. As the JM domain has been shown to contain several autoantibody epitopes, determining relationships between the JM domain epitopes and those lying within the central PTP region will be important for our understanding of epitope spreading within the IA-2 molecule.

7.2  Aim:

This study makes use of four mouse monoclonal antibodies to the juxtamembrane domain of IA-2 and three monoclonal human antibodies to defined epitopes within the central PTP domain of the molecule to study relationships between epitopes within the JM and PTP domains. The four monoclonal mouse antibodies used in this study recognize JM domain epitopes similar to those recognized by human type 1 diabetic samples (Ananieva-Jordanova, Evans et al. 2005; Piquer, Valera et al. 2006)
The specific aims were:

1. To determine which amino acids were important for binding of the mouse monoclonal antibodies to the JM domain and investigate whether these are similar to those recognized by JM antibodies in human type 1 diabetic patients.
2. To determine if there was any relationship between antibody reactivity to the JM and reactivity to the PTP domain by competitive binding studies.

7.3 Methods:

7.3.1 Patient sample selection.
Patients used in this study were taken from the same panel as 6.3. Of the 140 patients genotyped and analyzed for IA-2 antibody reactivity, 12 were selected for use in this study. All samples were defined according to reactivity to two domains of the IA-2 molecule known to contain major antibody epitopes: the JM domain and the PTP domain. Three serum samples were selected which recognized only the JM domain of IA-2, 4 which recognized both the JM and PTP domains, and 5 which did not recognize the JM domain.

7.3.2 IA-2 reactive antibodies:
This study made use of four mouse monoclonal antibodies, 76F, 5E3, 8B3 and 9B5. Three mouse monoclonal antibodies to the JM region (5E3, 8B3, 9B5) were sourced from RSR Ltd. (from Dr. J. Furmaniak, Dr. M. Powell and Dr. B. Rees-Smith, Cardiff), one (76F) came from CRT Dresden (from Dr. E Bonifacio), and the rabbit polyclonal antibody (R2B2) was developed by immunization with IA-2 biotinylated protein, by Professor A. Gardas, in Warsaw (Dromey, Weenink et al. 2004). Three human B cell clones 96/3, M13 and DS329 which are known to produce antibodies directed to known epitopes within the PTP domain of the IA-2 molecule were used (Kolm-Litty, Berlo et al. 2000; Ananieva-Jordanova, Evans et al. 2005; Weenink, Lo et al. 2009).

7.3.3 Antibody purification:
Monoclonal antibodies were purified by protein A-Sepharose chromatography. 0.5ml of Protein A Sepharose was swollen in D$_2$O for 30 minutes. The beads were then equilibrated by washing with 5mls of 100mM Tris, pH8, followed by 5ml 10mM Tris pH8. One tenth of the final volume of tissue culture supernatant from EBV transformed cell lines was added to tissue culture supernatant and the resulting mix was filtered through a 0.45µM syringe filter prior to adding to protein A-Sepharose. Supernatants were rolled with protein A-Sepharose overnight, prior to washing in 5mls of 100mM Tris, pH8.
Antibodies were eluted from protein A-Sepharose by adding 500µl 100mM glycine, pH3, and incubating for five minutes. This was repeated 10 times. All ten fractions were neutralized by adding 50µl 1M Tris, pH10 and mixing.

7.3.4 Preparation of Fab fragments:

Fab fragments for use in antibody competition studies were prepared by papain digestion. Papain (from papaya latex, Sigma, UK) was activated by adding 10mM cysteine, diluted in 1.25mM EDTA. Papain was then incubated for 15 minutes at 37°C, prior to addition to IgG at a concentration of 5-10 mg/ml, using a 1:50 ratio of enzyme to antibody. Antibodies were incubated with activated papain at 37°C overnight. Papain activity was inhibited by addition of 50mM iodoacetate. Fab fragments were then dialyzed overnight into PBS (pH7) using Slide-a-Lyzer dialysis cassettes with a 3000kDa cutoff (Peirce, UK). Fc fragments of the cleaved antibodies were removed by rolling with protein A-Sepharose overnight at 4°C.

7.3.5 Analysis of binding of IA-2 antibodies

Antibody binding to radiolabeled IA-2 constructs was analyzed by radioligand binding assay, as previously described in section 6.3.3. IA-2 constructs used were the cytoplasmic domain of IA-2 (IA-2ic, residues 605–979), a chimeric construct representing the juxtamembrane domain (JM, residues 606–700) fused to the tyrosine phosphatase (PTP) domain of PTP1B, the IA-2 PTP domain (residues 643–979) and the central region of the IA-2 PTP domain (residues 643–937). The inhibitory effect on antibody binding by addition of Fab fragments of individual antibodies were tested using analysis of variance.

7.3.6 Competition radioligand binding assays using Fab fragments of mouse monoclonal antibodies:

Fab fragments do not bind to protein A-Sepharose, so can be used in competition with whole antibody, in radioligand binding assays. Fab fragments were added at a concentration of 5µM in 5µl to serum samples in a standard RBA. Dilution was accounted for by adding an equivalent volume of PBS to serum samples. Samples were incubated overnight with 20 000cpm in 20µl of IA-2IC translate, then immune complexes were captured on protein A-Sepharose. Samples were washed through a filter plate, and bound radiolabeled IA-2 counted on a Chameleon scintillation counter (Hidex, UK).
7.4 Results:

7.4.1 Patterns of reactivity of mouse monoclonal antibodies: Peptide inhibition

All five antibodies were tested for JM reactivity using a standard radioligand binding assay, then tested for peptide inhibition using the system previously described for patient sera.

In contrast with polyclonal patient sera, each monoclonal antibody was only inhibited by one peptide, as seen in Figure 7-1 and one antibody, 9B5, was not inhibited by any of the peptides. The polyclonal rabbit antibody is inhibited by all three peptides, as 49% of patient sera.

![Figure 7-1: Peptide inhibition of mouse monoclonal antibodies; 76F was inhibited by p621-640, 5E3 by p611-630, 8B3 by p601-620 and 95 was not inhibited by addition of peptide.](image)

76F binding of the JM-chimera was significantly inhibited upon addition of peptide 621-640. 5E3 was inhibited by addition of peptide 611-630, and 8B3 by peptide 601-620. The 9B5 antibody was unaffected by addition of peptide, and the polyclonal rabbit serum was affected by all three peptides.

7.4.2 Definition of epitopes recognized by mouse monoclonal IA-2 antibodies using amino acid substitution

An in depth study of the epitopes recognized using site-directed mutagenesis demonstrated that mouse monoclonal antibodies recognize restricted epitope regions, with a requirement for amino acids 634-636. Figure 7-2 A shows data for the 76F mouse monoclonal antibody. This antibody is inhibited by the 621-640 peptide, and is inhibited by previously defined amino acids 626-629, 609 (cluster 6) (Bearzatto, Lampasona et al. 2003), and by the regions 616 and 632-636 (cluster 4). Figure
7-2 B shows 5E3, an antibody blocked by the 611-630 peptide only, with key amino acids groups being 615-621 and 633-638.

Figure 7-2 C shows the trace for 8B3, a JM1 reactive antibody, blocked by the 601-620 peptide. This antibody requires amino acids 611 and 615, and the common amino acids 634-638.

9B5 is not inhibited by any of the peptide blocking assays, but as seen in Figure 7-2 D, it requires a broad range of amino acids, reinforcing the idea the JM domain is a compacted loop: 9B5 recognizes amino acids 615,616, 626, 635- 639.

All four monoclonal antibodies were inhibited by amino acids from cluster 4 (615,616, 631,633-636) underlining the importance of this region for antibody recognition of the JM region.
Figure 7-2: Showing effects of single sequential substitution of amino acids from JM 608-JM 639 on mouse monoclonal IA-2 antibodies; A-D show data for four mouse monoclonal antibodies 76F, 5E3, 8B3 and 9B5.
7.4.3 **Inhibition of IA-2 human monoclonal antibodies by Fab fragments of IA-2 antibodies.**

To demonstrate direct inhibition of antibody binding of the PTP/central region epitopes, Fab fragments were also added to three central region antibodies and inhibition of binding was measured. As shown in Figure 7-3 there is partial inhibition of antibody binding for the central region reactive antibodies, 96/3, M13 and DS329, which are fully blocked by addition of M13 (central region reactive) Fab.

![Graph](image)

**Figure 7-3:** Inhibition of monoclonal antibody binding by addition of JM reactive Fab fragments.

Inhibition by addition of Fab fragments of PTP and JM domain-reactive monoclonal IA-2 antibodies was used to study the effects of competition for binding with monoclonal antibodies to IA-2. Addition of Fab fragments of the PTP domain autoantibody M13 prevented binding to other monoclonal antibodies recognizing similar PTP domain epitopes, but had no effect on IA-2 binding of the JM domain-reactive antibody, 76F, as can be seen in Figure 7-3. A rabbit polyclonal antibody to IA-2 was also unaffected by addition of PTP reactive Fab fragments. As expected, addition of Fab fragments of the JM domain antibodies abolished (5E3) or partially inhibited (9B5) IA-2 binding of the JM-reactive 76F antibody. However, Fab fragments of 5E3 and 8B3 JM reactive antibodies also partially inhibited IA-2 binding of the monoclonal antibodies M13, 96/3 and DS329 that recognize the PTP domain epitope. The results indicate that binding of Fab fragments of antibodies to the JM domain are able to impair antibody binding to epitopes within the PTP domain, possibly via steric hindrance or conformational effects.

7.4.4 **Inhibition of binding of IA-2 autoantibodies in Type 1 diabetic patients’ sera by Fab fragments of mouse monoclonal IA-2 antibodies**

This research group has previously shown a link between antibody recognition of the whole JM region, and T cell recognition of central region epitopes (McLaughlin, Gulati et al. 2014). This suggests some degree of interaction between the JM domain and the central region epitope. In
order to further investigate this pattern of reactivity in patient sera the inhibitory effects of Fab fragments of monoclonal antibodies were investigated using serum antibodies from IA-2 antibody-positive Type 1 diabetic patients. Addition of Fab fragments from the JM domain reactive antibodies abolished (5E3) or partially inhibited (8B3, 9B5) binding of antibodies from patients with reactivity restricted to the IA-2 JM domain, whilst addition of PTP reactive Fab fragments from M13 had no effect, as seen in Figure 7-4. Fab fragments of the JM domain antibodies also inhibited IA-2 binding of autoantibodies from patients recognizing both JM and PTP domain antibodies; and also those negative for JM antibodies, as seen in Figure 7-4. Inhibition of recognition of the central region by addition of Fab fragments of JM domain-reactive suggests structural interactions between these two regions of autoantibody reactivity.
Figure 7-4: A. shows inhibition of patient antibody binding by addition of Fab fragments, using JM only patients. B. shows inhibition of patients recognizing both JM and PTP epitopes. C. shows inhibition of non-JM reactive patient sera.

7.5 Discussion:

Each of the four mouse monoclonal IA-2 antibodies used in this study recognizes a different JM domain epitope. Using peptide inhibition to reveal the epitopes recognized by mouse monoclonal antibodies to the JM domain show that synthetic peptides shown to inhibit serum antibodies from Type 1 diabetic patients (601-620, 611-630 and 621-640 (Bearzatto, Naserke et al. 2002)) also inhibit binding of three of the mouse antibodies as shown in Figure 7-1. Site-directed mutagenesis demonstrates the importance of amino acids 615, 635 and 636 for antigen binding to all four monoclonal antibodies. Amino acids within the 608-638 region of IA-2 have different contributions to binding of individual antibodies: the 76F monoclonal antibody binding was affected by amino acids 626-629, which form part of the 621-640 and 611-630 epitopes described by the Bonifacio group (Bearzatto, Naserke et al. 2002; Lampasona, Belloni et al. 2008) whilst 5E3 requires residue 621, known to contribute to a 601-620 epitope (Lampasona, Belloni et al. 2008). All four mouse monoclonal epitopes recognized show similarities with the amino acid clusters recognized by type 1 diabetic subjects. As stated in Piquer et al (Piquer, Valera et al. 2006) several of the residues recognized by 76F are also key to patient sera recognition of JM epitopes (amino acids 609,626,636).

Fab fragments generated from JM reactive antibodies were more effective than those of PTP domain antibodies at blocking binding of serum antibodies to epitopes in both the JM and PTP domain. The inhibitory effects of JM reactive antibodies on binding of serum and monoclonal antibodies to the PTP domain is suggestive of close structural relationships between the two epitopes.

This study identifies several key amino acids, involved in the humoral immune response to a region important at the initiation of autoantibody response to IA-2. These key epitope regions are unique to IA-2; and may have some structural link with the central region of the protein, which could determine T cell reactivity to other regions of the molecule (McLaughlin, Gulati et al. 2014) and have implications for epitope spreading: an initial response to a specific epitope within the molecule can then lead to reactivity to other parts of the same molecule (Vanderlugt and Miller 2002). Epitope spreading is a critical part of the adaptive immune response, particularly in tumor clearance, with initial tissue damage leading to exposure to more tissue specific antigens and the development of a more targeted immune response. Whilst the link between the JM and central region remains unclear, recent work by this group suggests that enzymatic processing of IA-2 protein bound by JM antibodies leads to protection of peptides from both the JM and central region, which can then be
presented to T cells (McLaughlin, Richardson et al. 2015). Further studies should be performed to clarify any links with T cell peptides (McLaughlin et al, unpublished). Our group has shown previously that IA-2 841-860 peptide may provide a link with HLA-DR4. The association of antibody reactivity to specific JM domain epitopes shown in Chapter 6 suggests that there may also be T cell responses to JM domain peptides which are DQ8 restricted. Further defining these epitopes would allow characterization of the mechanisms underlying HLA-DQ8-mediated genetic susceptibility to disease.
Chapter 8  Conclusions

Type 1 diabetes is an autoimmune disease orchestrated by both T and B cells, underscored by specific genes. The disease affects millions of people, worldwide, with currently no cure. It is clear that a failure of immune tolerance is central to development of disease, but genetics, diet and infection also contribute, in a manner which is as yet unclear. Specific factors influencing disease development continue to be clarified. The central role of the T cell as the major orchestrator of disease onset, and view that the B cell only plays a peripheral role in the autoimmune response is no longer appropriate: B cells contribute to 30% of the islet infiltrate in the NOD mouse (Faveeuw, Gagnerault et al. 1995), and in the human, the degree of B cell infiltration of inflamed islets varies between individuals, from 1.6-21 cells per islet, in some patients B cells represent the second most abundant cell type in islet infiltrates after CD8 T cells (Willcox, Richardson et al. 2009) CD138+ plasma cells have also been found in islet infiltrates (Willcox, Richardson et al. 2009). The loss of B cell tolerance is one of the earliest warning signs for disease; antibodies to all of the major islet antigens, insulin included, are used as markers of at-risk individuals (Nokoff and Rewers 2013). Certain autoantibodies are associated with specific HLA types, HLA-DR3-DQ2 is most commonly seen in patients with GAD65 antibodies, whilst HLA-DR4-DQ8 is more commonly seen with Insulin antibodies and IA-2 antibodies (Ilonen, Hammais et al. 2013). Antibodies to ZnT8 are more commonly seen in individuals with the neutral DR13-DQB1*0604 haplotype (Salonen, Ryhanen et al. 2013).

As demonstrated by McLaughlin et al (McLaughlin, Gulati et al. 2014), and Weenink et al (Weenink, Lo et al. 2009), there are clear relationships between T and B cell responses. B cell autoimmunity is one of the earliest signs of disease (Gorus, Goubert et al. 1997): antibodies to the major islet antigens, Insulin, GAD65, IA-2 and ZnT8 can be detected many years prior to onset. The presence of multiple autoantibodies is a clear risk factor for disease (Ziegler, Rewers et al. 2013; Steck, Vehik et al. 2015). The six year results for the TEDDY study clearly demonstrate that many high risk HLA children develop autoantibodies to islet autoantigens (6.5%). Of these, 1.6% had only IA-2 antibodies. Each antibody type is associated with distinct HLA alleles and the degree of the antibody response is closely associated with the time of disease onset (Bingley, Bonifacio et al. 1994; Gottsater, Landin-Olsson et al. 1995).

The antibody response, general HLA associations and T cell response in type 1 Diabetes have been well characterized; the specific epitopes recognized by B cells, and HLA associations of these epitopes remain poorly understood. In order to re-establish tolerance, a clear understanding of the epitopes involved in B cell antibody binding and antigen presentation is necessary. Therefore, the
aims set out at the beginning of this study were to identify specific epitopes within the IA-2 molecule, using serum antibodies, monoclonal antibodies and monoclonal antibody fragments generated from single sorted IA-2 reactive B cells and investigate associations of antibody responses to specific epitopes with diabetes susceptibility genes within the HLA region.

To date, detailed knowledge of epitopes within the IA-2 autoantigen is restricted to the central region epitope (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009; McLaughlin, Gulati et al. 2014; McLaughlin, Richardson et al. 2015) its interaction with the C-terminus of the molecule, centering on the catalytic cysteine, C909 and (Elvers, Geoghegan et al. 2013) and the juxtamembrane domain (Lampasona, Bearzatto et al. 1996; Kolm-Litty, Berlo et al. 2000; Bearzatto, Lampasona et al. 2003).

This study uses recombinant monoclonal antibodies to the central domain to further study binding to this epitope region: two antibodies developed from monoclonal B cell lines, 96/3 and DS329 are capable of partially or fully inhibiting binding of sera to the central region epitope. This central region epitope is commonly recognized by human sera (Kolm-Litty, Berlo et al. 2000; Farilla, Tiberti et al. 2002). Recent studies proposed that the presence of anti-idiotypic antibodies to common, immunodominant epitopes prevent the development of type 1 diabetes (Oak, Gilliam et al. 2008): the 96/3 recombinant Fab fragment was used to demonstrate that this was not the case with the IA-2 molecule (Richardson, McLaughlin et al. 2013). The development of recombinant fragments of both of these molecules provides a simple way of inhibiting binding of the common central region epitope in polyclonal sera, allowing other less common epitopes to be uncovered. Binding of antibodies to other epitopes was also studied using the recombinant scFv generated from the 76F hybridoma cell line; which binds another commonly recognized epitope within the juxtamembrane domain of the molecule. The epitope for the 76F antibody has been characterized, results shown by Piquer et al (Piquer, Valera et al. 2006) were both confirmed, and further expanded, in chapter 7.

PCR amplification of IgG V-region genes from single sorted IA-2 positive B cells from a type 1 diabetic patients generated two fully functional antibody fragments, using the pFLAG vector to allow production of immobilized protein for direct binding assays and free protein to test inhibition of monoclonal antibodies and sera. Both scFvs were shown to be capable of both directly binding IA-2 and inhibiting monoclonal and polyclonal antibody binding to the molecule. Both antibody fragments were fully inhibited by substitution of the E836 amino acid, suggesting they bind a common conformational epitope in the central PTP region (Elvers, Geoghegan et al. 2013), as do 96/3 (Kolm-Litty, Berlo et al. 2000), M13 (Ananieva-Jordanova, Evans et al. 2005) and DS329 (Dromey, Weenink et al. 2004). Two more human monoclonal antibodies, isolated in Dresden, A6
and C11 (Catani, Walther et al. 2015) also recognize this epitope; there are subtle differences between all seven human monoclonal IA-2 antibodies now identified, yet the existence of a common epitope, recognized by all human monoclonal IA-2 antibodies isolated to date is surprising. Given the number of epitopes defined within the IA-2 molecule, and the polyclonal nature of the serum antibody response, more varied reactivities would be expected. However, of the seven monoclonal human antibodies generated six were generated from PBMCs; only C11 was generated from a peripheral lymph node (Catani, Walther et al. 2015). Understanding the nature of B cell reactivities at the site of inflammation may help our understanding of this question.

Recent advances in the extraction of intact RNA from FFPE tissues showed that amplification of preserved genetic material (Tyekucheva, Martin et al. 2015) was feasible. In 2005 Kent et al (Kent, Chen et al. 2005) demonstrated that it was possible to recover and expand T cells from pancreatic draining lymph nodes from type 1 diabetic and control patients. The group demonstrated more expansion of T cells in PLNs from diabetic subjects, and in HLA-DR4 individuals, many of these T cells recognized the insulin A 1–15 epitope. This data suggested that a study of B cells in pancreatic draining lymph nodes from archival tissue could yield interesting results. As a consequence of the successful generation of antibody fragments from single cells and cell lines, in chapter 4, mRNA was extracted from archival F tissue, from both type 1 diabetic patients and control tissues. Heavy chain sequences were successfully extracted from peripheral lymph node follicles from tissues from two type 1 diabetic patients. Future work to clone and express these sequences may allow determination of the target antigens of these proteins. Whilst fresh tissue from recent onset patients is difficult to come by, these preliminary studies demonstrate that it is possible to gain useful information from archival tissue.

To further examine specific epitope binding by monoclonal antibodies, this study used the NMR shift assay to attempt to clarify the already well characterized central region epitope, found within the PTP domain of the IA-2 molecule, focusing on the E836 amino acid (Dromey, Weenink et al. 2004; Weenink, Lo et al. 2009). The original aim of this section was to undertake a backbone assignment for NMR spectra of the PTP domain, and use this to identify amino acids affected by addition of a monoclonal antibody fragment. Unfortunately, low yields of the labelled target protein prevented this outcome, however, two dimensional spectra of the labeled protein, and protein complexed with scFv were generated. The spectra of PTP protein alone confirmed that the protein was stable at low concentrations for a prolonged period of time, distinct peaks could be visualized, and suggesting that the protein could be used to generate a backbone assignment for the PTP domain in the future. Data from this study show that with further optimization the scFv-PTP complex could be used to visualize
peak shifts without a dramatic loss of resolution. Unfortunately, although it was possible to see some shifts in specific peaks on addition of the scFv fragment, the PTP protein degraded rapidly on addition of the scFv preventing generation of any useful data about antibody binding.

The fifth section examines a less well characterized region of the molecule: the juxtamembrane (JM) domain, using site directed mutagenesis of a radiolabeled construct. The study clarifies the composition of previously defined juxtamembrane domain epitopes (Lampasona, Bearzatto et al. 1996; Bearzatto, Lampasona et al. 2003): The 601-620 epitope contains amino acids 611, 612, 618 and 619. The JM2 epitope is now shown to be conformational, not linear, with amino acids 609, 625, 626 and 627 contributing to its formation. All classes of autoantibodies required some or all of amino acids 615, 631, 633-636. The earliest autoantibodies to the IA-2 molecule to appear in pre diabetes are often those to the JM domain. Autoimmunity to the JM domain is associated with one of the major diabetes risk allele: HLA-DR4. However, there was evidence of heterogeneity in the autoimmune response to JM, suggesting that an in depth analysis of the epitopes in the JM domain was necessary. As with previous studies of the JM domain, this project identified two specific binding patterns, those sera which recognized JM 601-620, and those which did not. Alanine scanning mutagenesis further clarified epitopes within these groups, yielding six clusters of amino acids, which could be combined into four major groups.

1. Residues which had little or no effect on antibody binding with most sera
2. Residues which affected antibody binding in individuals with antibodies inhibited by the addition of a peptide representing amino acids 601-620.
3. Residues that contributed to antibody binding in patients with IA-2 JM antibodies unaffected by a peptide representing amino acids 601-620.
4. Residues required for binding of antibodies in most sera, irrespective of effects of the 601-620 peptide.

Data from this study demonstrate that as with central region antibodies, protein conformation is important for binding of serum autoantibodies to the JM domain of IA-2. Data generated through analysis of mouse monoclonal reactivity to IA-2 confirms this data, showing similar

Links with the high disease risk HLA-DR4 alleles are well established for the whole IA-2 molecule and the IA-2 JM domain (Williams, Aitken et al. 2008; McLaughlin, Gulati et al. 2014). This study establishes further links between HLA class II alleles and specific epitopes recognized within the JM domain (Chapter 6). I was able to demonstrate links between HLA-DR4-DQ8 and recognition of amino acids 621 and 622, defined as part of the JM 2 epitope. HLA-DR7 was linked to recognition of
the JM1 epitope. HLA-DR7 is reported to be a neutral risk allele for Type 1 diabetes, however, positive association of this allele with IA-2 autoantibodies has previously been reported (Williams, Aitken et al. 2008; Noble, Johnson et al. 2011). The association of B cell epitopes to DQ8 is most likely to be associated with DQ8-restricted T cell responses to as yet undefined T cell determinants with unknown peptide determinants. Possible candidates may include peptides located within the 601-633 region capable of stimulating T cell responses in HLA-DQ8 transgenic mice (Kudva, Deng et al. 2001).

It has been shown that B cell deficient NOD mice do not develop diabetes (Serreze, Chapman et al. 1996; Serreze, Fleming et al. 1998), and that depletion of B cells prevents the development of the autoimmune response in these animals (Fiorina, Vergani et al. 2008; Yu, Dunn et al. 2008; Zekavat, Rostami et al. 2008), and allotransplanted animals (Carvello, Petrelli et al. 2012). Although the B cell does not play an overt role in destruction of islet cells in the mouse (Bendelac, Boitard et al. 1988), they are key players, acting as highly efficient professional antigen presenting cells (Hulbert, Riseili et al. 2001; McLaughlin, Gulati et al. 2014) and have been show to play a key role in diversifying the immune response (Falcone, Lee et al. 1998). Antigen specific B cells are capable of binding and endocytosing antigen at low concentration via the high affinity B cell receptor, rapidly processing target antigen and presenting resultant peptides to T cells. The IA-2 specific antibody fragments generated in this study (Chapter 2 and Chapter 3) were used to study the binding of antigen: by attempting to clarify the presence of anti-idiotypic antibodies in circulation (Richardson, McLaughlin et al. 2013), and as an effective tool for characterizing antibody epitopes, by NMR and by competition studies in a radioligand binding assay (Chapter 5 and Chapter 6).

In summary, this study has shown that it is possible to extract V-region sequence from monoclonal B cell lines, single cells and fixed archival tissue. These sequences can be used to generate recombinant antibody fragments, which can be expressed in a bacterial system as functional proteins. Recombinant antibodies can be used to define epitopes recognized by antibodies in patient sera, and to study antibody binding of IA-2 epitopes (Richardson, McLaughlin et al. 2013). Both recombinant and serum antibodies were used to map epitopes within the central and juxtamembrane domains of the IA-2 protein, using site directed mutagenesis; this data was used to further define the links between central and juxtamembrane region epitopes (McLaughlin, Richardson et al. 2015). The data from this part of the study strongly suggests that the juxtamembrane domain and the central region epitope are closely aligned in the folded protein structure; this would allow JM specific B cells to process, and present peptides from the central region epitope: allowing diversification of the T cell and antibody response. Clearer definition of the
JM1 and JM2 epitopes, uncovering requirements for specific amino acids, together with links to HLA-DR7 and HLA-DQ8 gene expression will permit more specific targeting of IA-2 specific B cells. These results provide a clearer understanding of the role of antibody binding in type 1 diabetes; create a technique for generating functional autoantibody fragments from different sources, expressed easily and cheaply in bacteria. Further clarification of the structure and binding of both central region and juxtamembrane domain epitopes can be used to generate specific inhibitors of B cell binding to prevent or delay disease onset.
Chapter 9 References:


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