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Characterization of HLA-DQ8α/DQ2β Transgenic IAβ null NOD Mice as a Model for Type 1 Diabetes

by

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DEDICATION

I would like to dedicate this thesis to some of the most important people in my life both past and present. To my mom, thank you for your love and support through my 20 years of education. To my dad, thank you for the knowledge and wisdom you provided me that I carry with me through every step of my life. To Tim, thank you for your constant love, patience and understanding and for lending me your shoulder to lean on when times were tough. And finally to Rob, who was the reason I embarked on this journey.

ABSTRACT

Susceptibility to type 1 diabetes in humans is strongly associated with the heterozygous MHC class II haplotype DQ8/DQ2 (DQA1*301/B1*302, DQA1*0501/B1*0201). It has been hypothesized that the formation of DQ8α/DQ2β trans-heterodimers unique to these heterozygous individuals is responsible for the high risk associated with this haplotype. A DQ8α/DQ2β transgenic IAβ null NOD mouse line was established and this thesis describes the characterization of these mice as a potential humanized model for type 1 diabetes. The DQ transgenic mice developed severe insulitis and a small percentage progressed to diabetes. Since the DQ8α/DQ2β molecule is the only MHC class II restriction element present in these mice, they provide a useful model to examine responses to diabetes autoantigens and to map epitopes restricted to the DQ8α/DQ2β trans-heterodimer. By immunizing the mice with whole antigens and examining recall responses to overlapping sets of peptides, T cell epitopes were mapped for human proinsulin, IA-2 and GAD65. These mice provide a useful tool to gain further insights into the contribution of the DQ8α/DQ2β trans-heterodimer to diabetes autoimmunity in humans.

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ABBREVIATIONS

ABC	Avidin biotin complex
APC	Antigen presenting cell or Allophycocyanin
Asp	Aspartic acid
AUC	Area under the curve
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CDR	Complementarity determining regions
CFA	Complete Freund's adjuvant
CLIP	Class II associated invariant chain peptide
Con A	Concanavalin A
cpm	Counts per minute
CTLA-4Ig	Cytotoxic T-lymphocyte-associated antigen 4 immunoglobulin
DAB	Diaminobenzidine
D-MEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ER	Endoplasmic reticulum
EU	Endotoxin units
FACs	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GM-CSF	Granulocyte macrophage colony stimulating factor
GTT	Glucose tolerance test
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IA-2	Tyrosine phosphatase-like insulinoma antigen
IAA	Insulin autoantibodies
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif

kb	Kilobases
kDa	Kilodaltons
LPS	Lipopolysaccharide
MGH	Mouse growth hormone
MHC	Major histocompatibility complex
mU	Milliunits
NOD	Non-obese diabetic
OVA	Ovalbumin
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerytherin
RAG	Recombination activation gene
RNA	Ribonucleic acid
RPMI	Rothwell Park Memorial Institute media
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
SI	Stimulation index
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSST-1	Toxic shock syndrome toxin-1

CHAPTER I

GENERAL INTRODUCTION

The immune system is our body's defence system that protects us from invading pathogens and cancer. It is our internal security force composed of a large variety of different cells and molecules that are continuously surveying our bodies and launching attack on foreign invaders. In certain individuals, there is a break down in self tolerance and the immune system loses its ability to distinguish self from non-self. This triggers the immune system to attack self tissues resulting in a condition called autoimmunity.

Branches of the Immune System

The immune system consists of both specific and non-specific defence mechanisms. Innate immunity is the relatively non-specific component comprised of mechanisms of defence that are not specific for a certain pathogen. These include anatomical and physiological barriers, complement and certain immune cells such as phagocytes, natural killer cells, macrophages and dendritic cells. The cells in the innate immune system can bind to antigen using pattern recognition receptors. These receptors are specific to certain characteristics of broad classes of infectious organisms. Some of these receptors recognize patterns of bacterial lipopolysaccharide, peptidoglycan, bacterial DNA, dsRNA and other substances. The specific component of the immune system is called adaptive immunity and is capable of specifically recognizing and

selectively destroying certain pathogens. The adaptive immune system has immunological memory and displays a high degree of antigen specificity and recognition of self versus non-self.

Adaptive immunity is divided into two main branches: humoral responses and cell mediated responses. Humoral responses involve the interaction of B cells with antigen leading to the proliferation and differentiation of the B cells into antibody secreting plasma cells. Cell mediated immunity involves the generation of effector T cells. These include cytotoxic T cells that recognize and destroy target cells and T helper cells that secrete cytokines to help activate phagocytic cells, B cells and cytotoxic T cells (1).

The MHC Complex

The major histocompatibility complex (MHC) is a cluster of genes whose products play various roles in the function of the immune system. The MHC is a 3500kb region located on chromosome 6 in humans and is called the HLA complex (2). In mice, this region is called the H-2 complex and is present on chromosome 17. The genes of the MHC are divided into three groups; class I, class II and class III. In mice, the H-2 complex is composed of four major regions K, I, S and D. The K and D regions encode class I molecules, the I region encodes class II molecules and the S region encodes class III molecules. In humans, the class I genes are divided into three groups called HLA-A, HLA-B and HLA-C. In the middle of the MHC region is the class III genes and at the other end is the class II region which is subdivided into three smaller loci called DP, DQ and DR.

Class I genes encode integral membrane glycoproteins that are found essentially on all nucleated cell surfaces where they present antigens to CD8+ T cells. Class II genes also encode integral membrane glycoproteins, however they are primarily expressed on antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells where they present antigen to CD4+ T cells. Class III genes encode a diverse group of proteins including complement and inflammatory cytokines that play a variety of roles in the immune system (1,3,4). The work of this thesis pertains primarily to MHC class II, in particular HLA-DQ and is discussed in more detail below.

MHC Class II

MHC class II molecules are composed of an α chain with a molecular weight of 34kDa and a β chain with a molecular weight of 29kDa (5). MHC class II molecules consist of 4 domains, with the α1 and β1 domains forming the peptide binding groove and the α2 and β2 domains forming the membrane proximal region. MHC class II molecules are open at both ends of the peptide binding groove and typically bind peptides that are 13 to 25 amino acids in length (6,7). The MHC class II genes are co-dominantly expressed, so an individual will express both maternal and paternal alleles in the same cell. They are also highly polymorphic due to the presence of multiple alleles at each locus. In HLA-DQ and HLA-DP molecules, both the α and β polypeptide chains are polymorphic. One DQα (DQA1) and one DQβ (DQB1) gene is expressed and similarly one DPα (DPA1) and one DPβ (DPB1) gene is expressed. The DRβ chain is also highly polymorphic and the number of DRβ genes expressed can vary between 2 and 4 in different individuals. In contrast, there has only been one allele identified for the DRα

gene. The non-polymorphic DRα can be expressed in various combinations with the different DRβ genes (1,8). Additional HLA-DQ, DP and DRβ genes are located within the MHC locus, but are pseudo genes that do not express RNA or protein. It has been suggested that these pseudo genes may be involved in generating new alleles by gene conversion (2). The large number of different MHC class II molecules that can be expressed in a given individual increases the repertoire of peptides that can be bound and presented to CD4+ T cells.

Antigen Processing and Presentation on MHC Class II

Exogenous antigens from outside of the cell are typically processed and presented by MHC class II molecules on the surface of APCs. MHC class II molecules are synthesized in the endoplasmic reticulum (ER) where they bind a protein called the invariant chain in their peptide binding groove. Binding of the invariant chain stabilizes the MHC class II molecule and prevents endogenous peptides from binding in the groove. This complex is transported through the Golgi and into lysosomes where the invariant chain is degraded, leaving behind a portion of the chain called the CLIP (class II associated invariant chain peptide) associated with the peptide binding groove. Exogenous antigens taken up by the cells are degraded into short peptides in the lysosome. Within the lysosome, the CLIP is removed from the MHC class II molecule and an antigenic peptide is loaded into the groove. The peptide-MHC class II complex is transported to the plasma membrane where it can be recognized by CD4+ T cells (1,9).

The T Cell Receptor

The T cell receptor (TCR) has some structural similarity to immunoglobulin Fab fragments in that they consist of an α and a β chain each with an immunoglobulin-like variable (V) region and an immunoglobulin-like constant (C) region. The V region contains three hypervariable regions similar to the complementarity determining regions (CDRs) of immunoglobulin chains. A disulphide bond formed between cysteine residues in the constant region link the two chains of the TCR together. The transmembrane regions anchor the chains in the plasma membrane and contain positively charged residues that interact with the negatively charged residues of the CD3 signal transducing complex. The cytoplasmic tails of the CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) that interact with tyrosine kinases to initiate the signaling cascade for T cell activation.

The TCR does not recognize antigen alone, rather it recognizes processed antigen bound by self MHC molecules. TCR gene rearrangements create diversity so that the TCR can recognize a large number of processed antigens in the context of a smaller number of self MHC molecules. The variable regions of the TCR are encoded by rearranged VJ sequences for the α chain and rearranged VDJ sequences for the β chain. The constant regions of the TCR are encoded by a C gene segment. TCR gene rearrangements occur in the thymus during T cell maturation. Unproductive TCR gene rearrangements and selection cause a large majority of thymocytes to die by apoptosis before they get a chance to leave the thymus and enter the periphery. In the thymus, positive selection of thymocytes capable of binding to self MHC occurs resulting in

MHC restriction. Negative selection eliminates thymocytes that have a strong affinity for self MHC or self peptide-self MHC complexes leading to tolerance to self antigens.

In general, T cells can be subdivided into two different groups, CD4+ T cells and CD8+ T cells based on their expression of CD4 or CD8 coreceptors. These coreceptors increase the affinity of the bond between the TCR-peptide-MHC complex (1,10).

T Cell Activation

Activation and expansion of CD4+ T helper cells plays an essential role in the generation of both humoral and cell mediated immune responses. Antigen presenting cells present processed peptides bound to MHC class II molecules that are recognized by T helper cells. More specifically, an interaction between the TCR and the peptide-MHC class II complex leads to either activation and clonal expansion of the T cell or clonal anergy leaving the T cell in a state of non-responsiveness. The interaction of the TCR with the peptide-MHC class II complex is by itself not sufficient to activate naïve T cells, but rather this single interaction induces the cells to become anergic. Two distinct signals are required to fully activate naïve T cells. In addition to the interaction of the TCR-peptide-MHC class II complex, a second costimulatory signal is also required. The costimulatory signal involves the interaction between CD28 expressed on the surface of the T cell with a B7 molecule expressed on the APC. Dendritic cells have a relatively high constitutive level of B7 expression making them very potent activators of T cells. Other APCs such as macrophages and B cells must be activated to induce expression of B7 and are therefore less potent activators of T cells. These two signals together upregulate IL-2 production and expression of the high affinity IL-2 receptor leading to

proliferation and differentiation of the T cells into CD4+ effector T cells. Two distinct CD4+ effector T cell populations are generated which include the T_H1 and T_H2 subsets. T_H1 cells secrete IL-2, IFN-γ and TNF-β and are responsible for activating cytotoxic T cells (CD8+ effector T cells). T_H2 cells secrete IL-4, IL-5, IL-6 and IL-10 and are responsible for activating B cells leading to antibody production.

Cytotoxic T cells are generated by similar mechanisms as CD4+ effector T cells. The interaction of the TCR on naïve cytotoxic T cells with peptide-MHC class I in addition to the costimulatory signal leads to the production of small amounts of IL-2 and expression of the IL-2 receptor. Additional IL-2 generated from T_H1 cells is generally required for proliferation and differentiation of the naïve cytotoxic T cells. These activated cytotoxic T cells are then able to recognize and destroy target cells bearing the appropriate peptide-MHC class I complexes (1).

Type 1 Diabetes

Type 1 diabetes is a disease characterized by autoimmune destruction of the beta cells in the islets of the pancreas. A number of different cell types are present in the islets including glucagon secreting alpha cells, somatostatin secreting delta cells, pancreatic polypeptide secreting cells and insulin secreting beta cells. The most remarkable feature of type 1 diabetes histopathology is the specific destruction of beta cells within the islets with complete preservation of the other cell types. The large majority of cells within the islets are beta cells, so upon autoimmune destruction of these cells there is a loss of the islet architecture and in patients with diabetes the islets are very small or appear to be non-existent. Those islets that are seen are often infiltrated with immune cells, which is

referred to as insulitis. Beta cell destruction can begin years before clinical onset of the disease and histological studies suggest that symptomatic diabetes is seen only after a reduction in beta cell mass of about 80% (11). Destruction of the beta cells leads to insulin deficiency resulting in hyperglycemia and spontaneous ketosis. Patients require daily administration of insulin to control blood glucose levels and avoid diabetic ketoacidosis and even death. Despite the major advances in diabetes research and in the care of patients with the disease, morbidity and mortality statistics and the economic burden to the health care system remain high due to the high risk for complications including nephropathy, retinopathy, peripheral neuropathy, microvascular and macrovascular disease (12).

Diabetes Autoantigens

There are three major autoantigens associated with type 1 diabetes that have been identified by the high levels of autoantibodies directed towards these molecules circulating in the blood of pre-diabetic and diabetic patients. These autoantigens include insulin or proinsulin, glutamic acid decarboxylase 65 (GAD65) and tyrosine phosphatase-like insulinoma antigen (IA-2).

Insulin is the only autoantigen that is uniquely synthesized in beta cells of the pancreas. Insulin production first begins with the synthesis of a 110 amino acid precursor called pre-proinsulin. During translocation of pre-proinsulin into the ER, a 24 amino acid leader sequence is cleaved off to generate proinsulin. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Endopeptidases within the Golgi excise

the C peptide generating the mature form of insulin. Insulin, free C peptide and some proinsulin that remains intact or partially processed are packaged into secretory granules and are transported to the cytoplasm where they accumulate until released upon stimulation of the beta cell (13-15).

GAD is the enzyme involved in the synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) from glutamate and therefore plays an important role in the function of the nervous system. In humans there are two isoforms of GAD, a 65kDa form called GAD65 which is the predominant form associated with diabetes and a 67kDa form called GAD67. The two isoforms share a high level of homology except in the first 95 amino acids of the N-terminal region. The major site of GAD65 expression is the GABAergic neurons of the central nervous system, however it is also expressed at lower levels in the islets of the pancreas. GAD65 is expressed as both a soluble and membrane bound form. It has been suggested that localization of the enzyme to synaptic vesicle membranes may influence the visibility and exposure of the enzyme to immune cells upon exocytosis (16,17).

IA-2 is a protein tyrosine phosphatase-like protein that is predominantly expressed in neuroendocrine cells of the brain and pancreas. The catalytic domain present at the C-terminal end protrudes into the cytoplasm and the N-terminal domain is located within the secretory vesicles (18). Studies have shown that autoantibodies in the sera of type 1 diabetic patients react primarily with the cytoplasmic domain indicating that the major antigen determinants are located at the C-terminal end (19,20). The cytoplasmic domain contains five cysteine residues that are not present in other protein tyrosine phosphatases and it is suggested that disulphide bond formation is critical in

formation of the antigenic structure and conformational epitopes recognized by autoantibodies (21).

Diabetes Autoantibodies

A large number of recent onset diabetic patients have autoantibodies targeted towards various proteins, including those found in the islets, and it has been shown that autoantibodies can be present in the serum of patients years prior to the onset of clinical symptoms of the disease (ie. in the pre-diabetic period). Autoantibodies do not appear to play a role in autoimmune destruction of the beta cells, but they can act as important markers of beta cell autoimmunity (22). Numerous long term studies assessing autoantibodies in diabetic and non-diabetic patients have been done to determine if autoantibodies can be used to predict the development of type 1 diabetes in non-diabetic individuals. The frequency of these autoantibodies in the general population is quite low, however studies of recent onset diabetics reveals that at the time of onset 70-90% of patients are positive for islet cell autoantibodies. More specifically, these studies revealed insulin autoantibody frequencies of 43–69%, GAD65 autoantibody frequencies of 52–77% and IA-2 autoantibody frequencies of 55–75% at the time of diagnosis (23-32). It should be noted that anti-insulin antibodies appear in virtually all patients once they begin injecting exogenous insulin. If the patients are lucky enough to receive a successful islet transplant, these anti-insulin antibodies disappear during the time they are off insulin only to reappear once they start back on insulin. In general, the greater the number of different types of these autoantibodies an individual has the higher the risk that they will develop type 1 diabetes (33-35). The ability to predict type 1 diabetes in people

is of great importance because with early diagnosis and treatment, various measures can be taken to try and halt or decrease the rate of beta cell destruction and prevent complications.

HLA Associations with Type 1 Diabetes

Type 1 diabetes is a multifactorial disease influenced by both genetic and environmental factors. Studies conducted with twins has provided some of the strongest evidence for genetic predisposition to the disease. In identical twins, if one twin has diabetes the other twin has a 33% risk of developing the disease. In HLA-identical siblings the concordance rate is 15%, however in siblings that do not share the same HLA identity the concordance rate is only 1%. In particular, the highly polymorphic HLA-DR and DQ genotypes influence both susceptibility and resistance to diabetes. The strong linkage disequilibrium associated with these genes has made it difficult to assess the extent of their individual roles in diabetes development. Linkage disequilibrium is seen when the observed frequencies of haplotypes in a population does not agree with haplotype frequencies predicted by multiplying together the frequency of individual genetic markers due to the non-random assortment of alleles at neighbouring loci. This means that certain alleles are inherited together with a large frequency. Examples of this include DR3/DQ2 and DR4/DQ8.

A large percentage of people with type 1 diabetes have either the DR3 or DR4 alleles. DR1, DR8 or DR16 also increases susceptibility to type 1 diabetes, whereas DR11 and DR15 are protective for the disease. The strongest association with susceptibility and resistance to diabetes however is with particular HLA-DQ genotypes,

which can be further influenced by the HLA-DR subtype. Susceptibility to type 1 diabetes in humans has been associated with DQ8 (DQA1*301/DQB1*302) with a relative risk of 8-12 and DQ2 (DQA1*0501/DQB1*0201) with a relative risk of 3-5. The highest risk for diabetes is associated with the heterozygous combination of DQ8 and DQ2 with a relative risk of 5-35. In contrast, DQ6 (DQA1*0102/DQB1*0602) is associated with dominant protection from the disease. Even when a high risk allele is present such as DQ8 in combination with DQ6 the relative risk for disease development is less than one. (36-40).

HLA-DQ Trans-Heterodimers

The heterozygous DR3/DQ2,DR4/DQ8 genotype is most strongly associated with type 1 diabetes (36-40). Studies have shown that this genotype imparts a much higher relative risk for the disease than simply the sum or product of the relative risks associated with the single haplotypes (ie DR3/DQ2,DR3/DQ2 and DR4/DQ8,DR4/DQ8). This observation suggests a role for trans-heterodimer formation that may be responsible for the increased risk (41). Cis-heterodimers occur when the DQα chain encoded on one chromosome pairs with the DQβ chain encoded on the same chromosome, whereas trans-heterodimers are formed when the DQα and DQβ chains from opposite chromosomes pair together (Figure 1.1). The trans-heterodimers and cis-heterodimers may bind different peptides or bind certain peptides differently which may explain the increased risk associated with the DR3/DQ2,DR4/DQ8 genotype (42). Two-dimensional gel-electrophoretic analysis and HPLC peptide-map analysis of HLA molecules from DR3/DQ2,DR4/DQ8 heterozygous patients identified both cis and trans-heterodimers

(43). It has been difficult to determine for certain in this genotype which combination, DQ8α/DQ2β or DQ2α/DQ8β is associated with the strongest susceptibility. DQ8α/DQ2β is encoded in other genotypes associated with a high risk for diabetes development, whereas the other trans-heterodimer (DQ2α/DQ8β) is encoded in certain heterozygous genotypes which are not at high risk for diabetes giving some indication that the DQ8α/DQ2β molecule may be the predisposing molecule for high risk (41,44).

Influence of HLA on the Generation of Autoreactive T cells

HLA-DQ molecules associated with diabetes may be involved in the cascade of events that lead to the production and activation of autoreactive T cells. The steps involved in the development of autoimmunity can include the release of autoreactive T cells from the thymus, amplification and activation of these T cells in the periphery and failure of immune regulatory mechanisms that suppress these autoreactive T cells (45).

HLA molecules present on thymic APCs bind self peptides which are presented to T cells in the thymus. Negative selection involves the deletion of T cells that have TCRs with high affinity for self peptide-MHC complexes, leaving those with moderate affinity to mature and be released from the thymus into the periphery. Thymic selection of T cells on diabetes susceptible HLA molecules can lead to the production of T cells predisposed to autoreactivity. For example, the DQ8 molecule has a unique peptide binding pocket that interacts with four anchor residues located at positions 1, 4, 6 and 9 on the peptide and determines the MHC-peptide binding affinity. Peptides with large aliphatic residues at position 4 and negatively charged residues at position 9 bind with high affinity to the DQ8 molecule. During selection in the thymus, DQ8 molecules may

bind self peptides with similar motifs that form a less stable MHC-peptide interaction. Any T cells with TCRs that have low or moderate affinity for this complex would be released from the thymus into the periphery. In the periphery, these T cells may encounter the self peptide or a very similar peptide that has the particular motif with a large aliphatic residue at position 4 and a negatively charged residue at position 9 that can form high affinity interactions with the DQ8 molecule. According to some authors, the TCR-peptide-MHC interaction would be enhanced by the overall stability of the peptide-MHC complex and T-cell activation could occur based on the peptide-induced change in stability of the DQ8-peptide complex (45).

Amplification of these potentially autoreactive T cells in the periphery may occur by stimulation of the TCR through a variety of mechanisms. For example, viral or bacterial superantigens can act to non-specifically amplify large numbers of T cells. Amplification may also occur through chronic exposure of T cells to autoantigens or by mechanisms of molecular mimicry whereby microbial or viral components possess antigenic determinants similar to the autoantigen or can complex with certain HLA molecules (45). For example, infection with rubella virus or coxsackie B4 virus has been associated with an increased risk for disease development and virus specific T cells have been shown to be more prevalent in patients with type 1 diabetes than non-diabetic subjects (46,47). Another possibility is that with the highly susceptible DR3/DQ2,DR4/DQ8 genotype, the alpha and beta chains can complex in different combinations to produce four different DQ heterodimers. This increases the number of different susceptible DQ molecules that can cause amplification of a greater subset of

autoreactive T cells which could potentially achieve the threshold for progression of autoimmunity (45).

Another step in the progression of autoimmunity involves the activation of autoreactive T cells at the tissue site. Activation of T cells at these sites depends on numerous factors including antigen concentration, HLA density, expression of costimulatory molecules and the production of inflammatory cytokines and chemokines. Additionally, failure of regulatory mechanisms such as the deficient activation of regulatory T cells that normally function to keep the immune system in check and modulate these autoreactive T cells can lead to autoimmunity (45).

Environmental Factors Influencing Susceptibility to Type 1 Diabetes

Less than 10% of individuals with increased genetic susceptibility for type 1 diabetes progress to overt disease suggesting a role for environmental modification in the disease progression. Viral infections, dietary factors in early infancy, vaccination, climate, toxins and stress are all environmental agents that may increase the risk of type 1 diabetes in those individuals already genetically predisposed to the disease. Whether these factors promote development of the disease may be dependent on the timing of exposures to these environmental agents. In particular, recent evidence has provided a strong link between the initiation of beta cell destruction and perinatal exposure to viruses and certain types of foods. Recent findings have suggested a link between rubella embryopathy and enterovirus exposure during fetal development that may initiate autoimmunity and diabetes development. Other immune events such as maternal-fetal blood group incompatibility, pre-eclampsia in the mother, short gestational age, caesarean

section and neonatal respiratory disease have also been suggested as factors that may lead to a higher risk for diabetes development later in life. Exposure to certain food components such as nitrosamine components, cow's milk protein and gliadin has also been suggested to play a role in the initiation of beta cell destruction (48-51).

Non-Obese Diabetic Mice

The non-obese diabetic (NOD) mouse was originally established in 1980 by Makino *et al* (52). Research has suggested that T cells mediate the development of diabetes in the NOD mouse. Evidence for this stems from experiments showing that depletion of T cells by neonatal thymectomy or administration of immunosuppressive agents such as anti-CD4, anti-CD8, anti-CD3 antibodies or cyclosporin can prevent diabetes in these mice (53-56). Furthermore, the disease can be transferred by adoptively transferring T cells from diabetic NOD mice into immunodeficient strains of NOD mice such as NOD-SCID and NOD-RAG-/- mice (57,58).

Many mechanisms regulating immune responses have been shown to be defective in NOD mice. Some of these mechanisms include defective elimination of autoreactive T cells due to the upregulated expression of anti-apoptotic proteins, ineffective suppression of effector T cells due to decreased numbers of regulatory T cells, decreased activation-induced cell death and inefficient costimulation resulting in impaired induction of anergy in autoreactive T cells (59).

The NOD mouse expresses the MHC class I molecules K^d and D^b. They express a single MHC class II molecule called IAg7 and a deletion in the Ea promoter results in a lack of expression of IE molecules (59). IAg7 is characterized by a non-Asp residue at

position 57 of the beta sheet and a wider peptide binding groove than other MHC class II molecules (60). K^d, D^b, IAg7 expression and the absence of IE expression are all necessary for diabetes development in the NOD mouse. NOD IAβ null mice that do not express IAg7 do not develop diabetes. The NOD MHC by itself however, is not sufficient for diabetes development as shown by studies with MHC congenic strains of mice (61).

Both central and peripheral tolerance mechanisms may be defective in the NOD mouse. The association of class II molecules with autoimmune diabetes reflects the ability of these molecules to present peptides in a manner that fails to eliminate autoreactive CD4+ T cells in the thymus and activates them in the periphery. Studies have suggested that IAg7 forms weak, unstable interactions with peptides resulting in a failure to eliminate or inactivate autoreactive T cells (62). Alternatively, the unusual structure of IAg7 may allow it to bind and present certain peptides that cannot be presented by other MHC haplotypes (63).

Disease pathogenesis in the NOD mouse begins with immune cell infiltration of the islets. APCs including macrophages and dendritic cells first infiltrate peri-vascular ducts and peri-islet regions followed by infiltration with T cells and B cells resulting in peri-insulitis. This initial stage of peri-insulitis occurs in both male and female NOD mice. The later stages which involve a more invasive insulitis and destruction of beta cells in the pancreas primarily occurs in females resulting in a much higher incidence of diabetes in females than in males, however various environmental influences can affect diabetes incidence in both male and female NOD mice. Interestingly, in humans this gender bias is not seen. The three autoantigens that have been shown to be important in

diabetes in humans (proinsulin, GAD and IA-2) are also associated with diabetes autoimmunity in the NOD mouse (64-66).

The NOD mouse has contributed substantially to elucidating the pathogenesis of autoimmune diabetes. However, disease mechanisms in NOD mice do not necessarily mimic those in humans and many preventative therapies and treatments for autoimmune diabetes found to be effective in NOD mice have failed to be effective in humans. It is important to remember that the genes mediating the disease such as the MHC genes are mouse rather than human and caution must be taken when research in the NOD mouse is translated to the human disease.

Transgenic Mice and Type 1 Diabetes

Mice expressing transgenes for various cytokines, costimulatory molecules, disease associated MHC molecules, TCRs and autoantigens have played an important role in the study of many autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, autoimmune thyroiditis, systemic lupus erythematosus, autoimmune myocarditis and autoimmue diabetes.

Some studies have shown that diabetes onset can be accelerated in NOD mice when cytokines such as IL-10 are transgenically expressed in the beta cells under the control of the rat insulin promoter, however diabetes is prevented upon systemic administration of IL-10 (67,68). Some other studies have shown that transgenic expression of TNF-α locally in the islets of adult NOD mice protects against diabetes, however neonatal expression of TNF-α in islets accelerates diabetes development (69,70). Transgenic expression of anti-inflammatory cytokines such as IL-4, IL-12p40

homodimer and TGF-β in beta cells has been shown to inhibit the development of diabetes in these mice (71-73). Transgenic expression of the costimulatory molecule B7-1 in beta cells of NOD mice accelerates diabetes in these mice, but not when expressed in a genetically non-susceptible strain of mice (74). NOD mice expressing mouse proinsulin under control of the MHC class II promoter are protected from diabetes development (75). Other studies in which diabetogenic K^d-restricted beta cell-specific TCR transgenes were expressed in NOD mice accelerated the onset of diabetes, however when this transgene was expressed in NOD-RAG-2-/- mice so that the mice had a monoclonal T cell repertoire they developed diabetes later and less frequently (76).

Since each individual person expresses a number of different MHC class I and II molecules it is difficult to study the effects of individual MHC alleles in humans without the influence of other MHC alleles. The creation of HLA transgenic mice has allowed for the study of individual HLA molecules. A number of different transgenic mouse lines have been produced that express diabetes susceptible human MHC class II molecules including DQ8, DQ2, DR3 and DR4 and the diabetes resistant allele DQ6 on various backgrounds including NOD, C57BL/6 and C57BL/10. In one study, DQ8 and DR3 HLA transgenic mice created in the C57BL/6 background in the absence of endogenous mouse class II and expressing the costimulatory molecule B7.1 in the beta cells of the pancreas under the control of the rat insulin promoter developed diabetes, however no diabetes occurred in the mice when mouse class II was replaced with a non-susceptible diabetes HLA molecule such as DQ6 (77). DQ8, DQ2, DR3 and DR4 HLA transgenic mice have also been created on the NOD background in the absence of endogenous mouse class II. None of these HLA transgenic mice expressing only transgenes for

diabetes susceptible alleles in the absence of mouse class II have spontaneously developed diabetes. Despite this, these mice have provided valuable tools for mapping epitopes for diabetes associated autoantigens restricted to these human HLA class II molecules. GAD65 and proinsulin epitopes have been identified for DQ8 and DR4 and IA-2 epitopes have been identified for DQ8 (78-84).

Experimental Design

The main goal of this thesis was to create a humanized mouse model for type 1 diabetes and to specifically study the DQ8α/DQ2β trans-heterodimer associated with a high risk for the disease. We created mice transgenic for DQ8α/DQ2β in the absence of endogenous mouse class II in the NOD background. We chose to create mice expressing the DQ8α/DQ2β trans-heterodimer since some studies have provided evidence that this particular trans-heterodimer may be the predisposing molecule for increased risk of diabetes development compared to the other DQ2α/DQ8β trans-heterodimer. HLA-DQ expression and general immune function of these mice is described in Chapter III. Characterization of these mice as a model for type 1 diabetes is discussed in Chapter IV. This includes the examination of insulitis, diabetes development and autoantibodies in the mice. The DQ8α/DQ2β transgenic IAβ null NOD mice also provide a useful model to investigate target autoantigens and epitopes associated with type 1 diabetes in humans. Chapter V discusses the mapping of T cell epitopes of diabetes autoantigens including human proinsulin, GAD65 and IA-2. Mice were immunized with recombinant antigen and sets of overlapping synthetic peptides were used to define DQ8α/DQ2β-restricted immunodominant epitopes for the autoantigens.

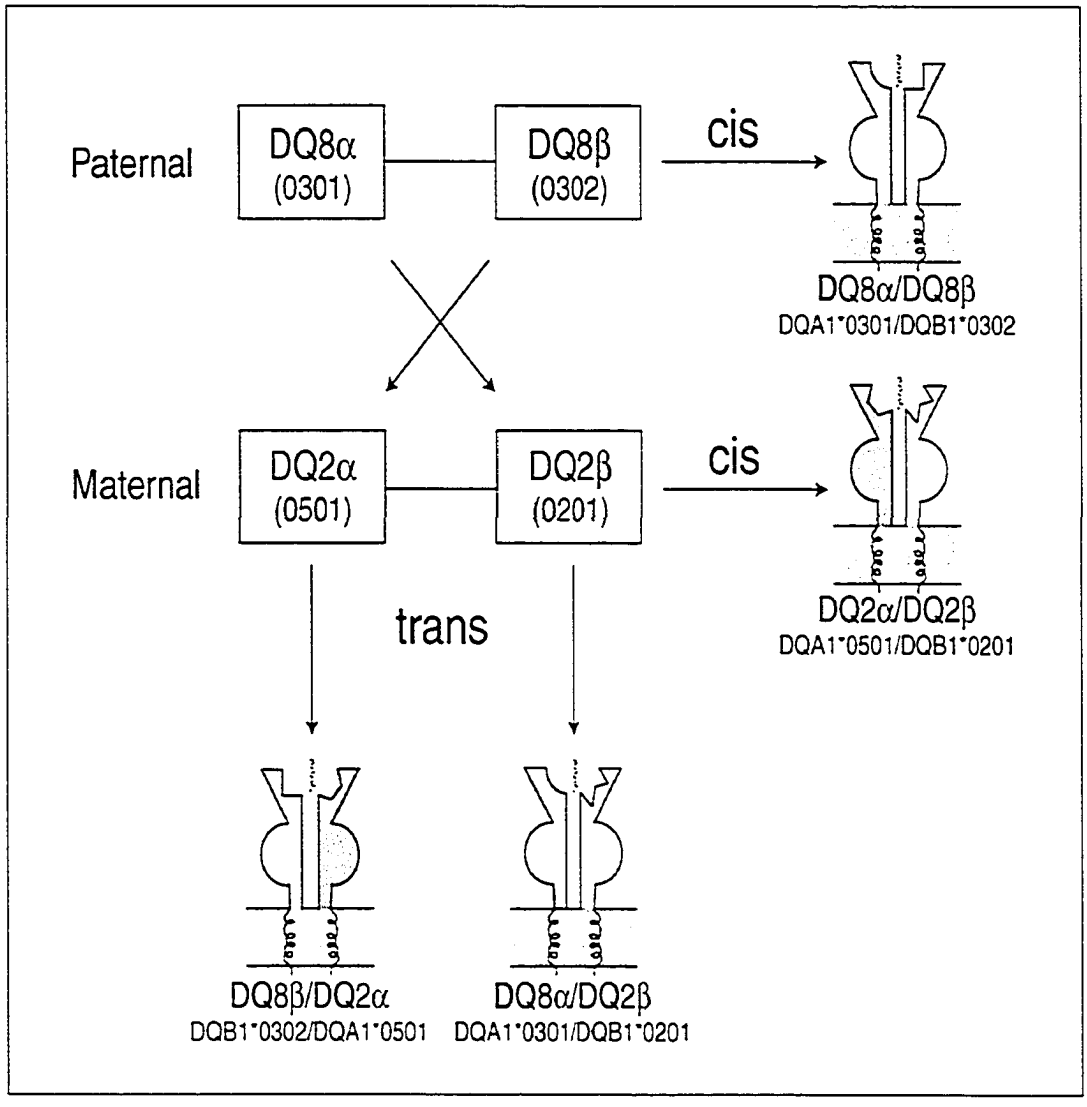


Figure 1.1. Cis and trans dimerization of HLA-DQ molecules for the HLA-DQ8/DQ2 haplotype. HLA-DQ α and β chains can pair in cis, where the DQα chain encoded on one chromosome pairs with the DQβ chain encoded on the same chromosome or in trans, where the DQα and DQβ chains from opposite chromosomes pair together. Cis and trans-dimerization produces four distinct HLA-DQ heterodimers. Adapted from: Type 1 Diabetes: Molecular, Cellular and Clinical Immunology. Chapter 1. Primer Immunology and Autoimmunity. Eds. G.S. Eisenbarth and K.J. Lafferty http://www.uchsc.edu/misc/diabetes/eisenbook.html.

CHAPTER II

MATERIALS AND METHODS

1. Mice

Separate human genomic inserts encoding DQA1*301 and DQB1*0201 and a human CD4 minigene construct (85) were microinjected into NOD IAβ null single cell embryos to produce the DQ8α/DQ2β IAβ null NOD mice. Founders with good DQ expression as determined by FACs were bred to homozygosity. To produce the DQ6 IAβ null NOD mice, a human genomic insert encoding DQA1*0102 and DQB1*0602 and the same human CD4 minigene construct were microinjected into wildtype NOD single cell embryos to first produce DQ6 NOD mice. These mice were then crossed with NOD IAβ null mice. F1 mice were intercrossed and F2 mice were screened by FACs. Mice with good DQ expression and no mouse class II expression were bred to homozygosity. Human genomic fragments were cloned from primary BAC genomic libraries by Dr. Alexey Atrazhev and Dr. John F. Elliott. Inserts were purified on sucrose gradients by Dr. Alexey Atrazhev and microinjection of the inserts into single cell embryos was done by Dr. Peter Dickie. NOD/LtJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. NOD IAβ null mice were supplied by Dr. Tony Jevnikar, University of Western Ontario. This line was created by backcrossing IAβ null mice created in the 129 background onto NOD/LtJ mice through 11 generations. BALB/cCr//AltBM mice were purchased from HSLAS, University of Alberta.

2. Proteins and peptides

GAD65, GAD65 recdel, human proinsulin and IA-2 were made in *E. coli* and purified over nickel chelating columns by Dr. Nurlan Kildibekov in the Elliott Lab. OVA was purchased commercially from Sigma. Overlapping peptide sets were purchased commercially from Chiron Mimotopes (Clayton, Australia). For GAD65, a set of 58 overlapping peptides were synthesized as 20mers overlapping by 10 residues. For the IA-2 cytoplasmic domain, a set of 74 overlapping peptides were synthesized as 15mers overlapping by 10 residues and for human proinsulin a set of 25 overlapping peptides were synthesized as 15mers overlapping by 12 residues. To confirm the GAD65 epitopes that we identified using the peptide set described above, we purchased a new set of peptides including GAD65 peptides 9, 11 and 28 from the NAPS Unit Peptide Synthesis Laboratory, University of British Columbia. Additional versions of GAD65 peptide 21 were synthesized in the Elliott Lab as a 20 amino acid version (P20) and a 14 amino acid version (P14), the latter consisting of only the 14 C-terminal amino acids of the P20 version. Other peptides that were used included; L18 23-42 (ribosomal protein), Derp-1 110-130 (dust mite allergen) and IA-2 920-934 all synthesized in the Elliott Lab, purified by HPLC and confirmed by mass spectrometry.

3. Endotoxin assay

GAD65, GAD65 recdel, human proinsulin and IA-2 proteins were tested for endotoxin using a Limulus Amebocyte Lysate QCL-1000 kit (BioWhittaker Inc).

4. Purification and PCR of murine genomic DNA

Mice were poked at the end of the tail with a 26 gauge needle and 75-100μL of blood was collected. DNA was isolated from the blood using a QIAamp DNA Blood Mini Kit (Qiagen). DNA from human blood samples including K (positive for DQ8α/β and DQ2α/β) and CC (negative for DQ8α/β and DQ2α/β) were used as controls. The concentration of DNA was determined by measuring the absorbance at 260nm. PCR reactions were completed on a PTC100 Programmable Thermal Controller using an allele specific priming method developed by Dr. Mike Bunce (86). The following was added to each well of a Thermowell 96 well V-bottomed PCR plate (Costar): 2.5μL 10x Platinum Taq buffer (Invitrogen), 0.5μL 10mM dNTPs, 0.75μL 50mM $MgCl_2$, 12.0μL sterile milliQ water, 0.2μL 5U/μL Platinum Taq polymerase (Invitrogen), 5μL 9μg/mL primers and 5μL of 10ng/μL genomic DNA. The mixture was covered with 10μL of mineral oil. The primers used were as follows:

DQ alleles	Primer Sequence	PCR Fragment Size
DQ8β (DQB1*0302,*0307)	GTGCGTCTTGTACCAGATA and TGGCTGTTCCAGTACTCGGCGG	121
DQ8β (DQB1*0307)	CCCGCAGAGGATTTCGTGTA and CCCCAGCGGCGTCACCA	156
DQ8α (DQA1*03011,*03012)	TTCACTCGTCAGCTGACCAT and CAAATTGCGGGTCAAATTCTTCT	183
DQ2β (DQB1*0201)	GTCCGGTGGTTTCGGAATGA and TGCTCTGGGCAGATTCAGAT	700
DQ2β (DQB1*0201,*0202,*0203)	GTGCGTCTTGTGAGCAGAAG and CGTGCGGAGCTCCAACTG	198
DQ2α (DQA1*05011,*05012,*05013,*0502)	ACGGTCCCTCTGGCCAGTA and AGTTGGAGCGTTTAATCACAC	189
Mouse Growth Hormone	GCCTTCCCAACCATTCCCTT and TCACGGATTTCTGTTGTGTTTC	428

Cycling parameters were as follows: 4 cycles of 94°C for 60sec, 94°C for 20sec, 70°C for 45sec, 72°C for 25sec, 20 cycles of 94°C for 25sec, 65°C for 50sec, 72°C for

30sec, 3 cycles of 94°C for 30sec, 55°C for 60sec, 72°C for 90sec, 1 cycle of 20°C for 30sec. Following PCR amplification, 5μL of 36% glycerol/64% Orange G dye in TBE (0.089M tris base, 0.089M borate, 0.002M EDTA) was added to each sample prior to loading onto a 1% agarose gel with ethidium bromide. The gel was run in TBE at 80V until the dye front had traveled 4cm. PCR fragments were visualized under U.V. light

5. Purification of mononuclear cells from murine spleen

Mice were euthanized by CO_2 and doused in 70% ethanol. Under sterile conditions, the spleen was removed and placed in a small petri dish containing 3mL of PBS. The spleen was ground between the frosted edges of two glass slides. The slides were rinsed with 2mL of PBS and the rinse was collected in the petri dish. The splenocyte suspension was overlaid onto 5mL of Lympholyte-M (Cedarlane CL5031) in a 15mL conical tube at room temperature. The suspension was centrifuged at 1150g for 20min at room temperature with the brake off. The mononuclear cell interface was collected and placed into a clean 15mL conical tube containing 10mL of complete D-MEM media (5% FBS, 2mM glutamine, 100U/mL penicillin, 100U/mL streptomycin in D-MEM) and centrifuged at 560g for 10min. The supernatant was removed and the pellet was resuspended in 10mL of complete D-MEM media and centrifuged at 560g for 10min. The supernatant was removed and the pellet was resuspended in 2mL of complete D-MEM media. Mononuclear cells were counted using a hemocytometer.

6. Culturing bone marrow-derived dendritic cells

Mice were euthanized by CO_2 and doused in 70% ethanol. Under sterile conditions, the skin on the leg was opened up and the muscle around the bone was cut away. The femur was removed and placed in 5mL of RPMI 1640 media in a 15mL conical tube. This was repeated with the other femur. The femurs were placed in a small petri dish and the media was aspirated off. 70% ethanol was added to the petri dish to sterilize the femurs. The ethanol was aspirated off and replaced with RPMI 1640 media. While holding the femur with tweezers over a 15mL conical tube containing 10mL of RPMI 1640 media, a 23 gauge needle attached to a 1mL syringe containing RPMI 1640 media was placed into the center of the femur where the red bone marrow could be seen. The bone marrow was washed out of the femur into the 15mL conical tube. Cells were counted using a hemocytometer and then centrifuged at 2250g for 10min. The supernatant was removed and the pellet was resuspended in cytokine media (10% FBS, 10ng/mL GM-CSF, 1.6μL/mL Gentamycin, 10ng/mL IL-4 in RPMI 1640 media) at a concentration of $1x10^6$cells/mL. Cells were plated in 6 well tissue culture plates at $4x10^6$ cells per well with 4mL per well and incubated at 37°C and 5% CO_2 (day 0). After 24 hours (day 1), the media in each well was aspirated off and replaced with 4mL of cytokine media. On days 2, 4 and 6, the plates were gently shaken for 1min and half of the media was aspirated off and replaced with fresh cytokine media. On day 8, the cultures were stimulated with 10ng/mL TNF-α and 10ng/mL LPS. On day 9, the cells were harvested, washed and stained for FACs analysis.

7. Cell staining and FACs analysis

Mice were poked at the end of the tail with a 26 gauge needle and 50-100μL of blood was collected into a microfuge tube containing 200μL of heparin (Heparin Leo 1000i.u./mL) diluted 1:200 in PBSB (1% BSA in PBS). Heparinized blood samples were added to a 96 well round bottom plate and centrifuged at 200g using plate carriers for 5min at 4°C. The supernatant was removed by aspiration and red blood cells were lysed by adding 180μL of ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA pH 7.4) to each well. The samples were incubated at room temperature for 5min and centrifuged at 200g for 5min at 4°C. The supernatant was removed and this procedure repeated until the majority of red blood cells had been lysed in the mononuclear cell preparation. Mononuclear cells prepared from blood, mononuclear cells prepared from the spleen or dendritic cells were suspended in PBSB containing the appropriate primary antibodies and incubated for 30min at 4°C. The cells were washed twice with 180uL of PBSB followed by centrifugation and aspiration of the supernatant as above. In cases where biotin labeled primary antibodies were used, 20μL of PE labeled streptavidin (Biosource International Lot #2202) diluted 1:800 in PBSB was added to each well and incubated for 30min at 4°C. Cells were washed as described above. The cells were resuspended in 100μL of cold fixer (2% para-formaldehyde in PBS) and kept at 4°C until analysis using a FACScan (Becton Dickinson). Antibodies used for FACs included antibodies purified from hybridomas in the Elliott lab as follows: SPV-L3 (mouse anti-human DQ monomorphic) FITC and biotin labeled, 10-3-6 (mouse anti-NOD IAg7) FITC and biotin labeled, GK1.5 (rat anti-mouse CD4) FITC labeled, RA3-6B2 (rat anti-

mouse B220) FITC labeled and H57-597 (hamster anti-mouse TCRβ) FITC labeled. All antibodies purified in the Elliott Lab were used at a concentration of 0.05μg/μL. Antibodies purchased from Cedar Lane that were used for FACs included anti-mouse F4/80 PE labeled, anti-mouse MAC-1 FITC labeled, anti-mouse CD11c APC labeled and anti-mouse CD8a (Ly-2) FITC labeled. Commercially purchased antibodies were used at concentrations suggested for FACs analysis on the specification sheets for each lot of antibody.

8. ^{3}H-thymidine uptake assay

Mice were immunized subcutaneously in the belly with 100μg of OVA, GAD65, GAD65 recdel, human proinsulin or IA-2. The proteins (100μg/150μL PBS) were emulsified with an equal volume of Freund's Complete Adjuvant (CFA) (Cedar Lane) and the entire 300μL was injected. In some instances, mice were boosted every 14 days after primary inoculation (1 boost for OVA, 2 boosts for human proinsulin and IA-2) in the same manner except Freund's Incomplete Adjuvant (IFA) (Cedar Lane) was used. Seven to fourteen days following the final inoculation the spleen was removed and splenocytes were purified over Lympholyte-M as previously described. In the case of GAD65 recdel, an additional immunization method was used in which the mice were immunized in both hind foot pads, the base of the tail and subcutaneously in the belly with a total of 100μg of protein diluted in 150μL PBS and emulsified in 150μL of CFA. The mice were boosted 7 days later in the same manner except IFA was used. Seven days following the final inoculation the spleen and popliteal lymph nodes was removed and splenocytes were purified over Lympholyte-M as previously described. Cells were

resuspended in complete D-MEM media at a concentration of $0.5x10^6$cells/mL and 200μL of cell suspension was added to each well of a 96 well flat bottom culture plate (Costar). Cultures were set up in triplicate with various concentrations of whole protein or peptides and a concanavalin A (ConA) positive control and no antigen control. In some experiments, blocking antibodies were used at a concentration of 100μg/mL. Blocking antibodies that were used included FN81.1 (mouse anti-human DQ monomorphic) and 10-3-6 (mouse anti-NOD IAg7) both purified from hybridomas in the Elliott Lab. After 56 hours of incubation at 37°C and 5% CO_2, 0.5μCi of ^{3}H-thymidine was added to each well. After an additional 16 hours, the plates were harvested onto UniFilter-96 GF/C plates using a Packard Filtermate Harvester and allowed to dry for 2-3 hours. MicroScint scintillation fluid (Packard) was added to each well and counts per minute (cpm) was read on a Packard TopCount-NXT Microplate Scintillation and Luminescence Counter. Stimulation index was calculated by taking the average cpm of triplicate sample wells and dividing by the average cpm of the no antigen control wells.

9. Spontaneous diabetes development

Mice were monitored on a regular basis for the development of diabetes. When diabetes was suspected, urine glucose was measured using Stanbio Clini-3 Reagent Strips for Urinalysis. Diabetes was confirmed by measuring blood glucose levels using a One Touch Ultra blood glucose monitor and test strips (Lifescan Canada Ltd.) and was defined as two consecutive blood glucose measurements of ≥11.1mmol/L 24 hours apart or a single blood glucose measurement of >33.3mmol/L if the mice were very ill and needed to be terminated immediately.

10. High glucose murine diet

Mice were fed a high glucose diet and monitored for diabetes development. The mice had a constant supply of sugar cubes (Roger's Sugar Ltd., Vancouver B.C.) mixed in with their regular rodent chow and each mouse would consume approximately 1 sugar cube every 2 days. Mice were started on the diet at various ages from 4 weeks to >1 year old and were kept on the diet for 4 months.

11. Cyclophosphamide-induced diabetes

Mice received two intraperitoneal injections of cyclophosphamide (Cytoxan, Bristol Laboratories of Canada) at a dose of 350mg/kg 7 days apart. Blood glucose was measured every 7 days for 2 weeks following the first injection. Diabetes was defined as two consecutive blood glucose measurements of $\geq$11.1mmol/L 24 hours apart.

12. Histology

Tissues were removed from the mice and fixed in 10% buffered formalin (Fisher). Fixed tissues were embedded in paraffin, sectioned at 4.5μm and stained with hematoxylin and eosin (H&E) by Lynette Elder, Dr. Greg Korbutt's Lab.

13. Scoring islet infiltration

H&E stained mouse pancreas sections were examined under a light microscope and the degree of insulitis was graded blindly by Dr. David Rayner. The total number of islets in each section were counted and islets infiltrated with mononuclear cells were given a score of 1-3 based on the degree of infiltration. The scoring system used was as

follows: 1 for peri-insulitis (mononuclear cells surrounding the islets but no infiltration of the islet architecture), 2 for moderate insulitis (mononuclear cells infiltrating less than 50% of the islet structure) or 3 for severe insulitis (mononuclear cells infiltrating greater than 50% of the islet structure).

14. Immunohistochemistry

Tissues were removed from the mice and embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co.). Frozen sections were cut on a cryostat by Lynette Elder, Dr. Greg Korbutt's lab and stored at -20°C. Sections were air dried for 5min, fixed in dry ice cooled acetone for 3min and air dried again for 5min. The following steps were all performed at room temperature. Slides were blocked in 2% FBS in PBS for 20min. Following blocking, 1 drop of avidin solution (Vector) was added to each tissue section and incubated for 10min and then 1 drop of biotin solution (Vector) was added and incubated for 10min. Primary antibodies were diluted in 2% FBS in PBS and added to the slides followed by incubation for 1 hour. The primary antibodies used were as follows: anti-CD4 (1:100), anti-CD8 (1:100), anti-CD11b (1:500) all purchased from BD Pharmingen and anti-B220 (1:200) purified from hybridoma RA3-6B2 in the Elliott Lab. After incubation with the primary antibody, the slides were washed 3x 2min with 2% FBS in PBS. Secondary antibody (biotinylated anti-Rat IgG, Jackson ImmunoResearch Laboratories) was added at a 1:200 dilution for 30min. During the incubation, the ABC complex (Vector) was prepared and incubated for 30min. Following incubation with the secondary antibody, the slides were washed as above and the ABC complex was added for 30min. The slides were washed as above and DAB Peroxidase Substrate (Sigma) was

added. Slides were visualized under a light microscope until desired degree of staining was seen. The reaction was stopped by washing the slides in distilled water. The slides were counterstained with hematoxylin (Sigma), dehydrated for 5min in xylene and coverslipped with Permount mounting media (Fisher).

15. Measurement of pancreatic insulin content

Pancreas was removed from the mice, weighed, placed in a freezing vial and immediately frozen in liquid nitrogen. Samples were kept cold on ice throughout the experiment. Frozen pancreas was added to 5mL of cold azol (0.25% BSA in 2M acetic acid) and homogenized. Samples were sonicated for 20sec, incubated for 2.5 hours at 4°C and sonicated again for 20sec. The samples were centrifuged at 1000g for 25min at 4°C with the brake off. Following centrifugation, 4mL of supernatant was removed for the insulin assay. The remaining supernatant was discarded and 5mL of cold azol was added to the pellet. The samples were sonicated and centrifuged as above and 2mL of supernatant was removed and added to the first 4mL of supernatant that was collected for the insulin assay. 50μL of the sample was added to 2mL of neutralizing buffer (2.5% BSA in PBS pH 11) and the samples were kept frozen at -20°C until time of assay. Insulin was measured in a radioimmunoassay by the Department of Lab Medicine and Pathology, University of Alberta Hospital and the units of insulin per gram of pancreas for each mouse was calculated.

16. Intraperitoneal glucose tolerance tests

Mice were injected intraperitoneally with a solution of 15% D-glucose in PBS warmed to 37°C at 2g/kg following overnight fasting for 16 hours with water available. Blood was taken from the tail vein and blood glucose was measured immediately before the glucose challenge and after 2, 3, 4, 5, 10, 15, 30, 60, 120, 180 and 240min following the challenge. Data was plotted and the area under the curve calculated using GraphPad Prism 4 Software (GraphPad Software, Inc.).

17. Detection of autoantibodies by Western blot

Tissue and cell homogenates were prepared and the protein concentrations of the homogenates were determined using a BCA assay by Sarah Wallbank in the Elliott Lab. Homogenates and Biorad broad range SDS-PAGE standards were diluted in 6x SDS/sample buffer and boiled for 5min prior to loading at 25μg of protein per lane on a SDS polyacrylamide gel (10% separating gel/3.9% stacking gel). Electrophoresis was carried out at 100V for 90min using a Biorad Mini-Protean II Dual Slab Cell. Electroblotting was performed using a Biorad Mini Trans-Blot Electrophoretic Transfer Cell and nitrocellulose membrane in transfer buffer (25mM Tris HCl, 192mM glycine pH 8.0, 20% methanol) at constant 250mA for 1 hour. Blots were blocked in blocking buffer (5% skim milk powder, 0.05% Tween in PBS) for 1 hour at room temperature with shaking. Blots were rinsed once in PBST (0.05% Tween in PBS) and were incubated overnight with primary antibody (serum diluted 1:100 in blocking buffer). Blots were washed 3x 15min in PBST and then incubated with secondary antibody (goat anti-mouse HRP conjugated Fc antibody, Jackson ImmunoResearch Laboratories, diluted 1:2500 in

blocking buffer) for 1 hour at room temperature with shaking. Blots were washed as above and developed using ECL-Plus Western blotting detection system (Amersham Pharmacia Biotech UK Limited).

18. Measurement of mouse insulin autoantibodies

Mice were euthanized by CO_2 and blood was immediately collected by cardiac puncture with a 23 gauge needle and 3mL syringe. The blood was centrifuged at 2000g for 4min and the serum was removed for analysis. Samples were sent to Liping Yu (Barbara Davis Center, Denver, CO) for detection of mouse insulin autoantibodies using a Ninety-Six Well Filtration Plate Micro-IAA Assay. Samples were run with one high level and two low level positive controls and a negative control. The result was calculated based on the difference in cpm (Δcpm) between the well without cold insulin and the well with cold insulin and was expressed as an index: index = (sample Δcpm - negative control Δcpm)/(positive control Δcpm - negative control Δcpm). The limit of normal (0.010) was chosen as the 99th percentile from receiver operating characteristic curves in 106 healthy control subjects and 105 patients with recent onset diabetes.

CHAPTER III

EXPRESSION AND EVALUATION OF THE FUNCTION OF HLA-DQ8α/DQ2β MOLECULES IN NOD IAβ NULL MICE

INTRODUCTION

Transgenic mice created as models of human autoimmune diseases have provided valuable tools for investigating basic mechanisms underlying autoimmunity. HLA genes have been identified as some of the strongest markers of autoimmunity (44). Research in disease mechanisms underlying these genetic associations has been greatly enhanced by the expression of disease associated HLA alleles in humanized transgenic mouse lines.

In autoimmune diabetes in humans, certain HLA-DQ alleles are highly associated with the predispostion to diabetes development, in particular DQ8 (DQA1*0301, DQB1*0302) and DQ2 (DQA1*0501, DQB1*0201). The heterozygous combination of DQ8 and DQ2 in humans confers a high relative risk for diabetes development and studies have shown that this may be due to the formation of DQ8α/DQ2β trans-heterodimers (87). To study the DQ8α/DQ2β trans-heterodimer in an *in vivo* model and attempt to create a humanized model for autoimmune diabetes, we produced a transgenic line of NOD mice expressing the DQ8α/DQ2β trans-heterodimer in the absence of mouse IAβ as described in the Materials and Methods. These DQ8α (DQA1*301) DQ2β (DQB1*0201) IAβ null NOD mice will be referred to as DQ8α/DQ2β mice for the remainder of this thesis.

It has been suggested that in HLA transgenic mice there may be a problem with CD4 T cell selection in the thymus due to a poor interaction of the mouse CD4 molecules and the HLA–peptide–TCR complex (88). Therefore, we included a human CD4 transgene when creating the DQ8α/DQ2β mice. Unfortunately, we found that there was relatively low expression of the human CD4 molecule in the line we investigated. This should not pose a problem however, since many groups have shown that sufficient numbers of T cells selected on human HLA molecules in the thymus can be generated when only mouse CD4 is present (89,90).

The first step in determining whether these mice could provide a useful model for studying autoimmune diabetes was to evaluate the expression and function of the DQ8α/DQ2β transgenes. PCR of genomic DNA was used to confirm the DQ8α/DQ2β genotype of the mice. Immune cells from these mice were examined by FACs analysis to ensure that DQ is expressed on the surface of these cells in the absence of mouse class II. In order for the transgenic DQ molecule to function normally in these mice it must be expressed on the appropriate immune cell populations, so we examined DQ expression on B cells, macrophages, dendritic cells and T cells.

The composition and function of the immune system of DQ8α/DQ2β mice was examined and compared to NOD mice to ensure that replacing the mouse class II with the human class II did not change the immunological make-up of the mice. Populations of immune cells including CD4+ T cells, CD8+ T cells, B cells and macrophages were identified in splenocyte and peripheral blood populations from DQ8α/DQ2β and NOD mice by FACs. Cell populations in these mice were compared to determine if there were any differences between the two groups of mice, which has been observed by groups

studying similar mice (91). Immune system function was examined by determining whether DQ8α/DQ2β mice could show antigen specific recall responses comparable to responses shown by NOD mice. The specificity of the responses was confirmed through blocking studies using MHC class II antibodies specific for DQ and IA.

RESULTS AND DISCUSSION

Mice transgenic for DQ8α (DQA1*301) and DQ2β (DQB1*0201) were identified by PCR analysis of genomic DNA purified from white blood cells using allele specific primers as described in the Materials and Methods. A sample PCR analysis of the DQ8α/DQ2β transgenic mice is shown in Figure 3.1 and confirms that the mice are transgenic for DQ8α (DQA1*301) and DQ2β (DQB1*0201).

In addition to PCR which identified mice that had the DQ8α/DQ2β transgene, expression of the transgene in the absence of mouse class II was also examined in these mice. PBLs from the mice were double stained with two monoclonal antibodies, biotin-conjugated SPV-L3 plus streptavidin PE for DQ and FITC conjugated 10-3-6 for IA and analyzed by FACs. For the PBL analysis a gate was set that encompassed the lymphocyte population. Figure 3.2 shows sample FACs plots comparing staining of PBLs from DQ8α/DQ2β mice, NOD mice and an F1 cross of DQ8α/DQ2β and NOD mice. PBLs from DQ8α/DQ2β mice stained only with SPV-L3, NOD mice stained only with 10-3-6 and the DQ8α/DQ2β x NOD F1 cross stained with both SPV-L3 and 10-3-6. This method of FACs analysis was continuously used to screen new DQ8α/DQ2β mice following weaning to ensure expression of DQ in the colony remained stable over time.

MHC class II molecules are primarily expressed on antigen presenting cells, so we used FACs analysis to ensure that the DQ molecules were being expressed on the appropriate immune cell types. PBLs from DQ8α/DQ2β mice were double stained with biotin-conjugated SPV-L3 plus streptavidin PE and either FITC conjugated RA3-6B2 (anti-mouse B220) for B cells, anti-mouse MAC-1 for macrophages or H57-597 (anti-mouse TCRβ) for T cells and analyzed by FACs. DQ expression on dendritic cells was also examined, however due to the small numbers of dendritic cells in the blood a different method of preparation was used to generate substantial numbers of dendritic cells prior to FACs analysis. Monocytes were obtained from the bone marrow in the femurs of DQ8α/DQ2β mice. The cells were cultured for 8 days in media containing GM-CSF and IL-4 which allowed for the generation of all three lineages of myeloid cells including dendritic cells, macrophages and granulocytes. An important step in this procedure was to use a series of gentle washes over the 8 days of culture to remove the majority of non-adherent granulocytes, leaving dendritic cells and macrophages stuck to the bottom of the culture well. To allow the dendritic cells to mature, the cells were cultured for 1 day in media containing TNF-α and LPS prior to FACs analysis (92). The dendritic cells were double stained using biotin-conjugated SPV-L3 plus streptavidin PE and anti-mouse CD11c APC. The macrophages present in the culture do not stain due to the lack of CD11c on their cell surface. Granulocytes on the other hand do express CD11c which is why it was important to remove them with the washes while the cells were being cultured. Figure 3.3 shows that DQ is expressed on antigen presenting cells including B cells, macrophages and dendritic cells and is absent from T cells as expected.

Representative DQ8α/DQ2β mice and NOD mice were sacrificed and immune cell populations were characterized by FACs. Splenocytes and PBLs were isolated from the mice and single stained with FITC-conjugated antibodies for CD4+ cells, B cells (B220), CD8+ cells and a PE-conjugated antibody for macrophages (F4/80). In the peripheral blood, DQ8α/DQ2β and NOD mice had comparable numbers of CD4+ cells (44% vs. 42%) and macrophages (6% vs. 8%). DQ8α/DQ2β mice had a slightly higher percentage of CD8+ cells (17% vs. 10%) compared to NOD mice, whereas NOD mice had a slightly higher percentage of B cells (44% vs. 35%) compared to DQ8α/DQ2β mice (Figure 3.4). In the spleen, DQ8α/DQ2β mice had a slightly higher percentage of CD4+ cells (29% vs. 23%), CD8+ cells (16% vs. 8%) and B cells (45% vs. 36%) compared to NOD mice, whereas the number of macrophages were comparable (5% vs. 6%) (Figure 3.5).

The function of the immune system of DQ8α/DQ2β mice was evaluated by examining the ability of the mice to mount antigen specific recall responses. These responses were compared to responses shown by NOD mice. The method used to measure the amount of T cell stimulation was ^{3}H-thymidine incorporation into DNA, which reflects the amount of lymphocyte proliferation. DQ8α/DQ2β mice and NOD mice were immunized subcutaneously in the belly with 100μg of OVA emulsified in CFA. Fourteen days following the initial injection, the mice were boosted in the same manner except IFA was used. Seven days following the second injection the spleen was removed and splenocytes were purified. Proliferation of splenocytes incubated with various concentrations of OVA was measured and the results in Figure 3.6A show that

the immune system of DQ8α/DQ2β mice can function to give concentration-dependant, antigen specific recall responses similar to NOD mice.

Blocking studies were performed to ensure that recall responses to OVA observed in the DQ8α/DQ2β mice was to due the presentation of antigen to T cells by the DQ molecule. Proliferation of splenocytes incubated with OVA and either no antibody or with blocking antibodies for DQ and IA was measured. As shown in Figure 3.6B,C, proliferation of splenocytes from DQ8α/DQ2β mice was blocked by the anti-DQ antibody SPV-L3 and was not blocked with the anti-IA antibody 10-3-6. Conversely, proliferation of splenocytes from NOD mice was not blocked by the anti-DQ antibody but was blocked by the anti-IA antibody. This data indicates that proliferation of T cells from the DQ8α/DQ2β mice to OVA was due to the presentation of the antigen by the DQ molecule expressed on the surface of antigen presenting cells.

Figure 3.1. Determination of the HLA-DQ8α/DQ2β genotype in a transgenic mouse by PCR analysis. White blood cell DNA was purified and amplified using primers specific for DQ8β (DQB1*0302,*0307) (**A**), DQ8β (DQB1*0307) (**B**), DQ8α (DQA1*03011,*03012) (**C**), DQ2β (DQB1*0201) (**D**), DQ2β (DQB1*0201,*0202,*0203) (**E**) and DQ2α (DQA1*05011,*05012,*05013,*0502) (**F**). Primers specific for MGH were included as a positive control (**G**).

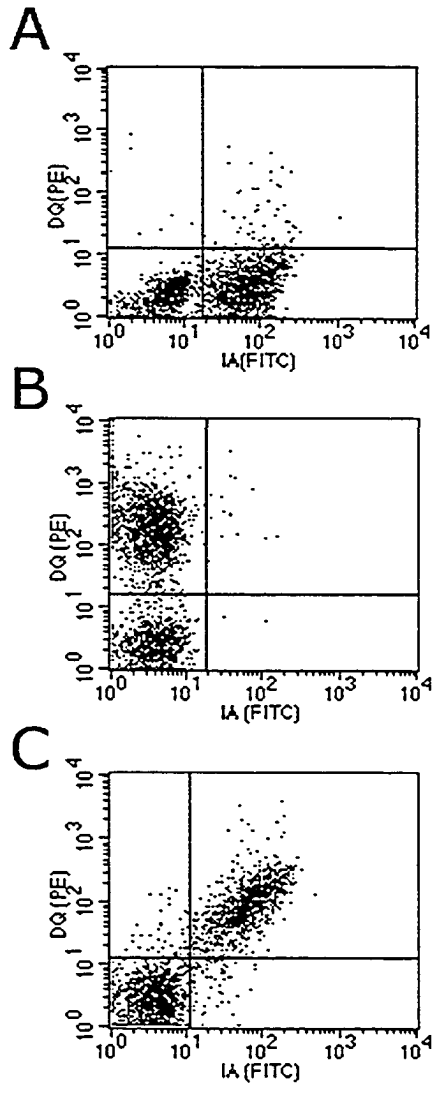


Figure 3.2. FACs analysis of HLA-DQ expression versus mouse class II in the transgenic mice. PBLs from the mice were purified and stained for DQ and IA with SPV-L3 and 10-3-6 antibodies respectively. Sample FACs plots for NOD mice (**A**), DQ8α/DQ2β mice (**B**) and DQ8α/DQ2β x NOD F1 cross (**C**) are shown.

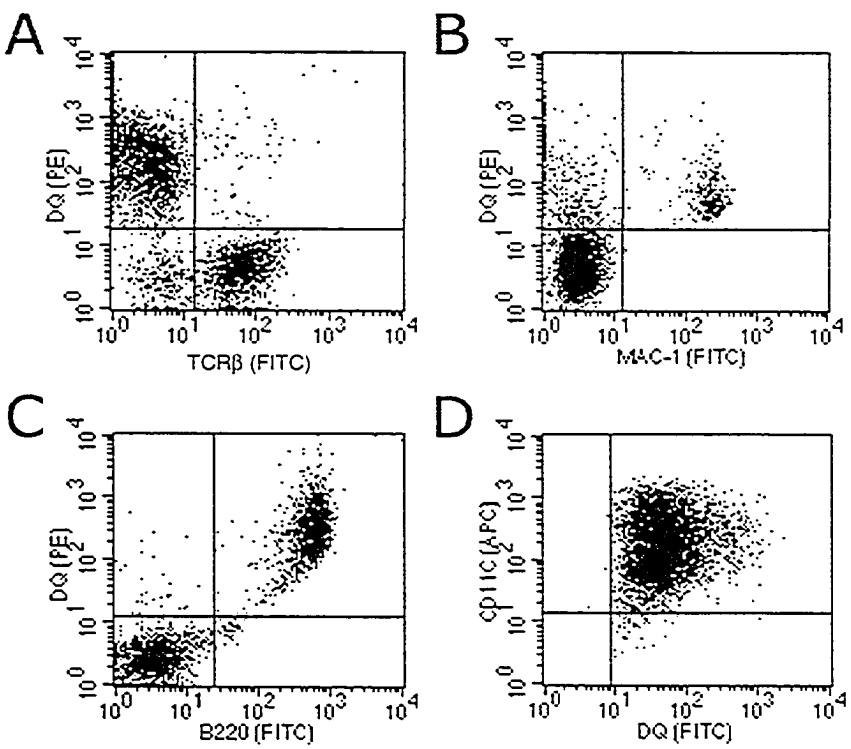


Figure 3.3. Determination of HLA-DQ expression on immune cell subpopulations by FACs analysis. PBLs from DQ8α/DQ2β mice were purified and double stained for DQ and either TCRβ (**A**), MAC-1 (**B**) or B220 (**C**). Dendritic cells from the mice were cultured and double stained for DQ and CD11c (**D**). Results are representative of four different DQ8α/DQ2β mice that were tested.

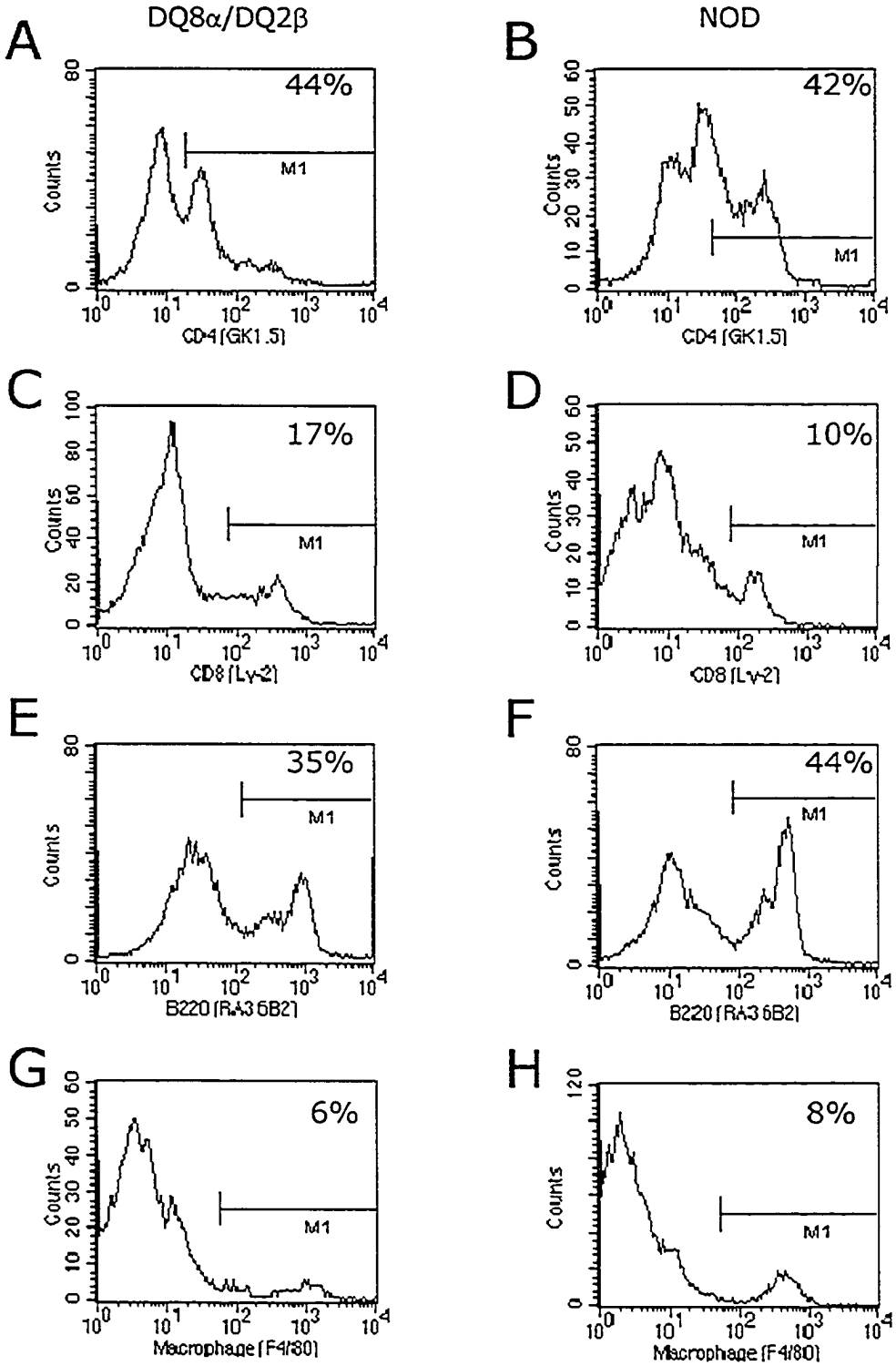


Figure 3.4. Comparison of subpopulations of immune cells in peripheral blood. PBLs from DQ8α/DQ2β mice (**A,C,E,G**) and NOD mice (**B,D,F,H**) were purified and stained for CD4 (**A,B**), CD8 (**C,D**), B220 (**E,F**) or F4/80 (**G,H**). Proportion of cells staining within the M1 gate are shown as percentages for each FACs plot.

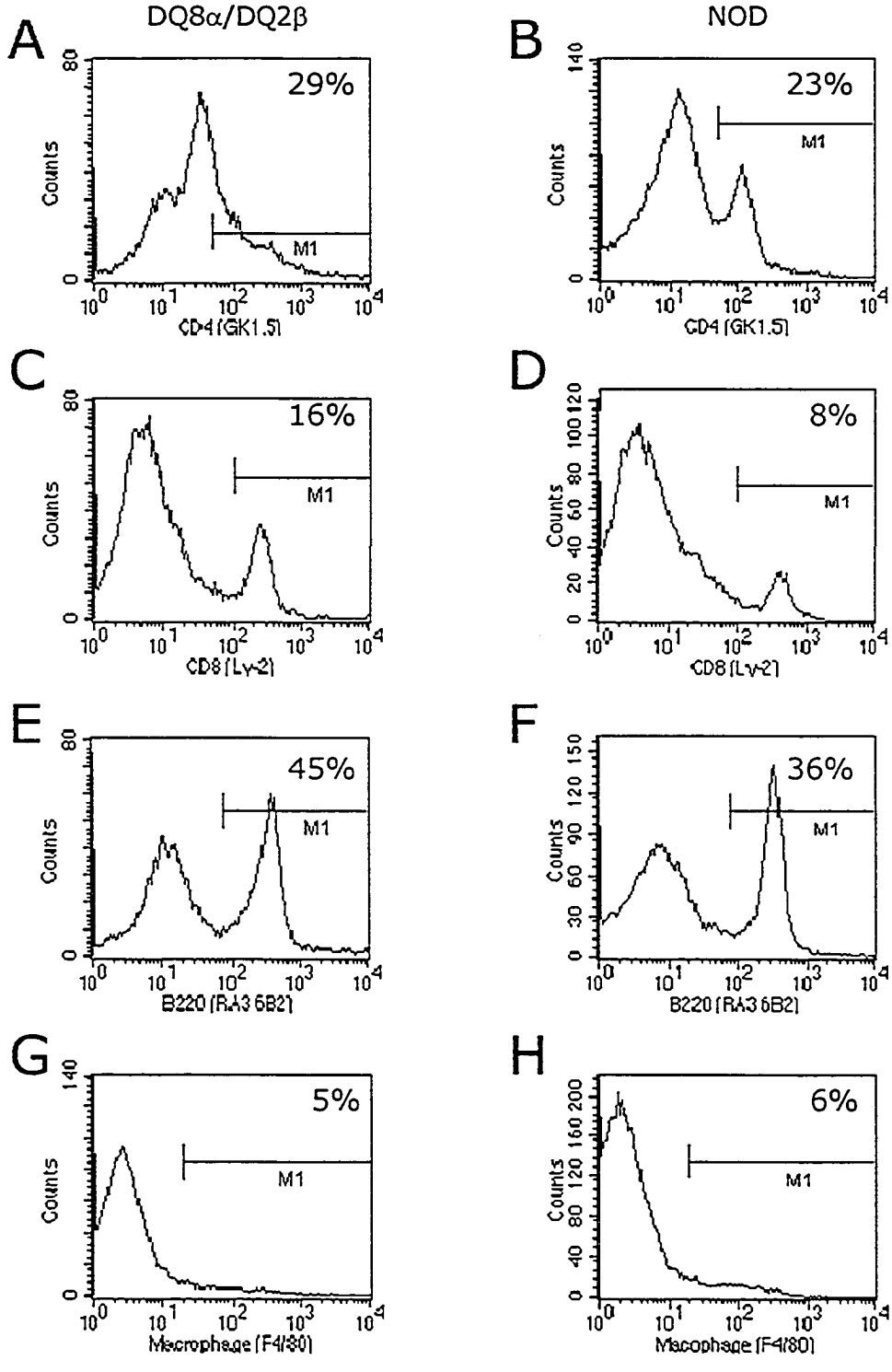
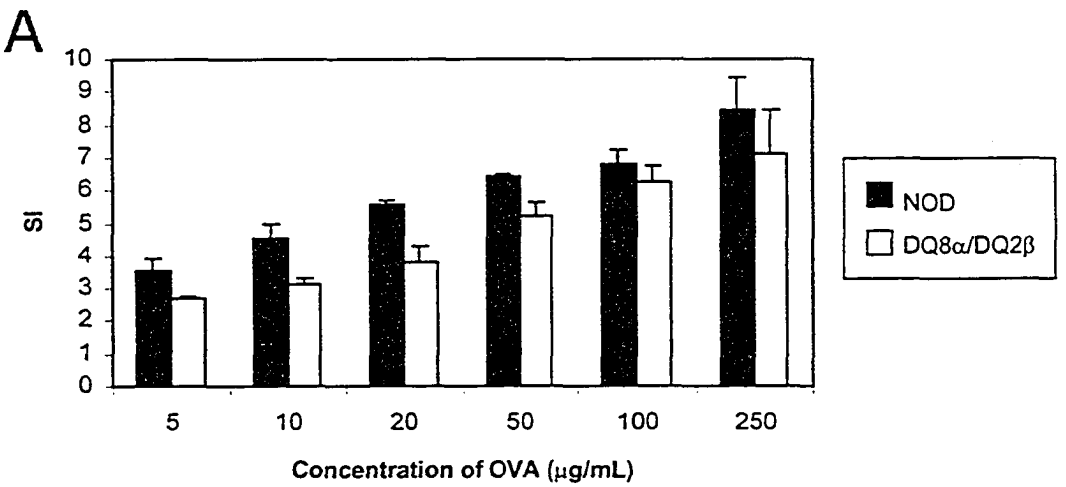


Figure 3.5. Comparison of subpopulations of immune cells in the spleen. Splenocytes from DQ8α/DQ2β mice (**A,C,E,G**) and NOD mice (**B,D,F,H**) were purified and stained for CD4 (**A,B**), CD8 (**C,D**), B220 (**E,F**) or F4/80 (**G,H**). Proportion of cells staining within the M1 gate are shown as percentages for each FACs plot.



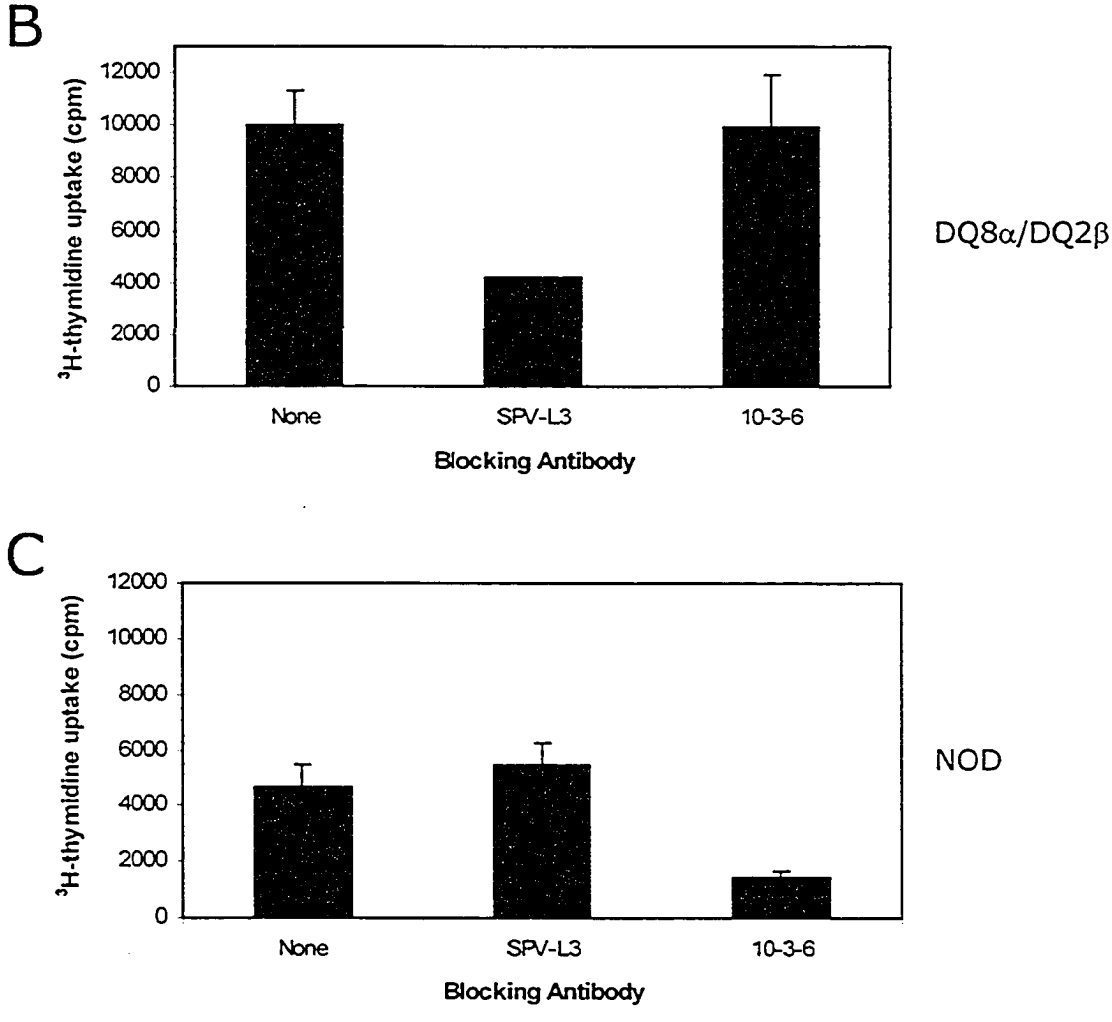


Figure 3.6. Demonstration of normal antigen presentation by HLA-DQ8α/DQ2β transgenic splenocytes. Splenocytes from OVA immunized mice were purified and proliferation of the splenocytes in the presence of various concentrations of OVA was measured in a ^{3}H-thymidine uptake assay (**A**). Splenocytes from OVA immunized DQ8α/DQ2β (**B**) and NOD (**C**) mice were incubated with 20μg/mL OVA and either 100μg/mL of SPV-L3 or 10-3-6 blocking antibody or no antibody and proliferation measured. Results are representative of two DQ8α/DQ2β mice and two NOD mice that were tested.

CHAPTER IV

EVALUATION OF INSULITIS, DIABETES AND AUTOANTIBODIES IN DQ8α/DQ2β IAβ NULL NOD MICE

INTRODUCTION

The ultimate goal in the creation of an animal model of disease is to develop a model in which the disease develops spontaneously with characteristics similar to the human disease. Autoimmune diabetes pathogenesis has been widely studied in the NOD mouse and has provided great insight into the etiology of the disease in humans, however the genes mediating the disease are mouse and not human. Many groups of researchers have expressed MHC class II molecules associated with diabetes in humans in various strains of mice including NOD, C57BL/6 and C57BL/10 mice in the absence or presence of endogenous mouse MHC class II in an attempt to create a more "humanized" model of the disease and to study the contribution of certain MHC class II molecules to the disease. HLA transgenic mice expressing diabetes associated alleles in the absence of mouse class II have not spontaneously developed diabetes (78). Some transgenic mice expressing DR3 and DQ8 in the NOD IAβ null background have been shown to develop insulitis, (93) although not by all groups studying these mice. In a study to map DQ8-restricted T cell epitopes for diabetes autoantigens, Dr. Elliott's lab created DQ8 IAβ null NOD mice (84). When the NOD mouse class II was replaced with the human diabetes-associated DQ8, the mice lost the autoimmune diabetes phenotype failing to spontaneously develop

insulitis or diabetes and instead developed autoimmune myocarditis (94). The DQ8α/DQ2β transgenic mice we have created are the first mice of their kind that transgenically express only human HLA molecules on the NOD IAβ null background that not only develop insulitis but also develop diabetes.

A large cohort of DQ8α/DQ2β mice were bred and monitored for spontaneous diabetes development. We also explored methods to accelerate progression of the disease. Cyclophosphamide, a drug that is primarily used to treat certain types of cancer, can also accelerate progression of diabetes in NOD mice, but not in other non-diabetic strains of mice (95). We were interested to see if cyclophosphamide could also induce diabetes in the DQ8α/DQ2β mice. We also tried challenging the mice with a high glucose diet in an attempt to accelerate progression of the disease.

Diabetes is the end stage of a disease that first involves initiation of mononuclear cell invasion of the islets followed by destruction of beta cells within the islets. When enough of the beta cells in the islets have been destroyed, the insulin deficiency is significant enough that glucose levels cannot be adequately controlled and diabetes develops. We explored a number of methods that could be used to monitor beta cell destruction and disease progression prior to overt diabetes. We visualized mononuclear cell invasion of the islets by histology and used immunohistochemistry to identify the infiltrating immune cell types. Pancreatic insulin content assays were used to quantitate the amount of insulin in the pancreas of mice at various ages and glucose tolerance tests were used to determine the ability of the mice to metabolize glucose.

The loss of beta cells in the islets is often associated with the appearance of islet autoantibodies in the serum. These autoantibodies are good predictors of disease risk as

they can often be detected in human serum many years before clinical onset of the disease (96). We used Western blotting to detect the presence of autoantibodies directed towards the pancreas and beta cells in the DQ8α/DQ2β mice by probing tissue extracts with serum from the mice. More specifically, we utilized the expertise of Dr. George Eisenbarth and the Ninety-Six Well Filtration Plate Micro-IAA Assay developed in his lab to detect the presence of insulin autoantibodies in the serum of our mice (97).

RESULTS AND DISCUSSION

The entire colony of DQ8α/DQ2β mice were continuously monitored for the development of diabetes. Initial indications of diabetes onset included a wet, sweet smelling cage caused by excessive urination and excretion of glucose in the urine due to disease development. The mice usually had a scruffy, wasted appearance and drank significantly more water than normal. At the first sign of a diabetic mouse, urine glucose was measured. Following a positive result for glucose in the urine, blood glucose was measured and diabetes was defined as blood glucose measurements of $\geq$11.1mmol/L for at least two consecutive days. In a few cases, when the diabetic mice were discovered they were extremely wasted and dehydrated and needed to be terminated immediately, so one blood glucose measurement of >33.3mmol/L was defined as diabetic.

Table 4.1 outlines the incidence of spontaneous diabetes in the DQ8α/DQ2β colony. The first diabetic mouse discovered on November 19, 2002, was a 27 week old male that was hemizygous for DQ8α and DQ2β. Over the next nine months, three more mice developed diabetes. All of these mice were male and homozygous for DQ. These three mice developed diabetes at 27, 42 and 64 weeks of age. It is interesting that all of

the DQ8α/DQ2β mice that developed diabetes have been males considering that in wildtype NOD mice it is the females that have a much higher incidence of the disease. Histopathology of the pancreas of these diabetic mice showed almost a complete loss of islets with only 1 or 2 residual islets remaining, some of which were infiltrated by lymphocytes. Additionally, pancreas sections from these mice did not stain for insulin. At the time that the fourth mouse developed diabetes, the incidence of diabetes in the colony showed that 4 out of 36 mice (11.1%) 25 weeks of age or older developed diabetes. Many young mice were taken out of the colony at various times to be used for experiments, so we only included mice that lived to be 25 weeks of age or older for our calculations.

Over the next year, no additional DQ8α/DQ2β mice developed diabetes, however they still developed insulitis. Included in this time was a period between January and August 2004, when the DQ8α/DQ2β mice bred very poorly. Very few litters were born during this time and of those breeder pairs that did rear pups, the pups were very runty and often did not survive past weaning. One possible suggestion as to why the incidence of diabetes in the DQ8α/DQ2β colony decreased so dramatically could be due to environmental influences. The rooms in the conventional area of the animal facility where these mice were housed were always positive for pinworms, however in July 2003, approximately one month before the last DQ8α/DQ2β mouse developed diabetes, the mice were moved to a new room in the facility that was pinworm positive and became suspect for parvovirus shortly after the mice were moved into the room. Exposure to parvovirus may have had an effect on their immune system that slowed down destruction of the beta cells and progression of the disease. Environmental factors can play an

important role in diabetes development. For example, diabetes incidence in NOD mice colonies reported by different institutions has been highly variable and a number of groups have shown that diabetes in NOD mice can be prevented by exposure to murine viruses such as encephalomyocarditis virus, lymphocytic chorio-meningitis virus and murine hepatitis virus (98). As reported by Suzuki *et al*, diabetes incidence in male NOD mice increased significantly from 6% to 70% when the mice were moved from a conventional room in the animal facility into germfree conditions, so it is likely that diabetes may decrease if mice were moved from a relatively clean room to a germ positive room like in the case of the DQ8α/DQ2β mice (99). This also raises the question as to whether or not diabetes incidence in the DQ8α/DQ2β mice may have initially been even higher if the mice were housed in a pinworm-free room. We attempted to embryo re-derive the DQ8α/DQ2β line so that the mice could be housed in a germ free environment to see if this would have any effect on diabetes incidence. However, due to the poor breeding of the mice our attempts to embryo re-derive the line were unsuccessful. This experiment is currently being repeated, but will not be completed in time to be included in this thesis.

In December 2003, approximately 4 months after the last DQ8α/DQ2β mouse developed diabetes we began mixing sugar cubes in with the regular rodent chow so that the mice had a constant supply of sugar. We hypothesized that if the mice were fed a diet high in glucose that maybe this would push the mice towards a higher incidence of diabetes. The entire colony of DQ8α/DQ2β mice were started on the high glucose diet at the same time. Therefore, mice were started on the diet at various ages anywhere from 4 weeks to over 1.5 years of age and as new mice were born they were started on the diet

immediately following weaning. After 4 months, no mice developed diabetes, so we stopped the high glucose diet and returned the mice to the regular diet of rodent chow.

Progression of diabetes in NOD mice can be accelerated by injections of cyclophosphamide. The drug does not induce diabetes in strains of mice not predisposed to diabetes indicating that diabetes development in the mice is not the result of toxicity to the pancreatic beta cells (100). We wanted to determine if cyclophosphamide could induce diabetes in the DQ8α/DQ2β mice. Mice were injected with two doses of cyclophosphamide 7 days apart. Following the first injection, blood glucose was monitored every 7 days for two weeks. As shown in Table 4.2, all of the female NOD mice developed diabetes and 3 out of the 8 males developed diabetes. No male or female DQ8α/DQ2β mice developed diabetes during the 2 week period following the injections. These mice were monitored for an additional 4 weeks and did not develop any signs of diabetes.

Due to the low incidence of overt diabetes in these mice we needed to develop a method of assessing disease progression. One method we explored was examining the histopathology of the pancreas. A complete histological analysis was done on a large cohort of DQ8α/DQ2β mice to assess the degree of mononuclear cell invasion of the islets. As a control, DQ6 mice (DQ6 IAβ null NOD mice) expressing the DQ allele that is protective for diabetes were included in the assessment. Male and female DQ8α/DQ2β and DQ6 mice were sacrificed at various ages and the pancreas was collected for histology. A grading system was developed to score islets for the degree of insulitis and pancreas sections were blindly scored by Dr. David Rayner. Islets were counted and given a score of 1 for peri-insulitis, 2 for moderate insulitis or 3 for severe insulitis

(Figure 4.1). The results of the histological analysis are presented in Table 4.3. Male and female DQ8α/DQ2β mice 4-8 weeks of age had a low percentage of islets with peri-insulitis at 1.3% and 1.6% respectively. None of the mice in this age group showed moderate or severe insulitis. The greatest amount of islet infiltration was seen in the 24 week age group of DQ8α/DQ2β mice. Female mice in this group had 15.4% of islets with peri-insulitis, 7.7% with moderate insulitis and 1.3% showing severe insulitis. Male mice in this group had 6.3% of islets with peri-insulitis, 12.7% with moderate insulitis and 3.8% showing severe insulitis. DQ8α/DQ2β mice in the 36 week age group showed considerably less islet infiltration than the 24 week age group. None of the mice in this age group had islets showing severe insulitis. Male mice in the 36 week age group did not show any signs of moderate insulitis, while female mice had a small percentage of islets with moderate insulitis (1.1%). Male and female mice 36 weeks of age had 2.5% and 12.7% of islets showing peri-insulitis respectively. None of the DQ6 mice in any of the age groups showed signs of insulitis.

One downfall of using histology to examine beta cell destruction is that the mice must be sacrificed for the experiment, so the same mice cannot be monitored over time. Histology provides a picture of the pancreas at one moment in time and it is impossible to determine if islets that were previously present in the pancreas have been destroyed. This may be one reason why the 24 week age group showed a greater amount of islet infiltration than the 36 week age group. Less insulitis may be seen at the later ages (ie. 36 weeks) because many of the islets have been completely destroyed and the lymphocytes have moved out of the area.

As mentioned earlier, the DQ8 mice (DQ8 IAβ null NOD mice) which were originally created as a potential model for diabetes developed autoimmune myocarditis instead, which was characterized by immune cell infiltration of the heart. We wanted to determine if this was also characteristic of the DQ8α/DQ2β mice so hearts were collected for histology from a small number of mice of varying ages. The histology showed no signs of infiltration in the hearts from these mice.

The H&E stained sections of the pancreas that were used for the histological assessment of the mice were useful for showing immune cell infiltration, but give no information about the types of immune cells that were infiltrating the islets. Therefore, we used immunohistochemistry to identify infiltrating cell types. Frozen pancreas sections from 24 week old DQ8α/DQ2β mice were cut and stained using monoclonal antibodies for CD4, CD8, B220 and CD11b. Figure 4.2 shows that the mononuclear cells infiltrating the islets consisted of a large number of B cells (B220 staining) and CD4+ T cells with slightly fewer CD8+ T cells and very few macrophages (CD11b staining).

Measuring the amount of insulin in the pancreas of the mice was another method we explored to assess disease progression. The hypothesis was that the insulin content of the pancreas should decrease with age as the beta cells in the pancreas of the mice are destroyed. For this experiment, the pancreas was collected from a cohort of male and female DQ8α/DQ2β mice at 6 and 38 weeks of age. The weight of the pancreas was measured and insulin was extracted using an acid extraction protocol. The samples were sent to the University of Alberta Hospital where the amount of insulin in the samples was determined using a radioimmunoassay. The amount of insulin per gram of pancreas was then calculated for each mouse.

The pancreatic insulin content of individual mice within the same age group was found to be quite variable as shown in Figure 4.3A,B. The results do give some indication of individual mice that may have had a greater degree of beta cell destruction compared to others. For example, mouse #1 in the 38 week age group had a very low insulin content compared to the rest of the mice in the group indicating that this mouse may have been headed towards diabetes. When the average pancreatic insulin content was calculated for each age group and the different age groups compared there was not a significant decrease in insulin between the 6 and 38 week age groups and the values were similar to those seen in 5 and 19 week old NOD IAβ null mice which do not show any signs of insulitis (Figure 4.3C). As with histopathology, the major drawback of measuring pancreatic insulin content as a method of assessing beta cell destruction is that the mice must be sacrificed for the experiment and therefore the same group of mice cannot be monitored over time.

The third method we explored to assess disease progression was glucose tolerance testing (GTT). GTTs can be used to measure the ability of the mice to metabolize glucose. The mice were fasted overnight and then injected with a solution of warm glucose intraperitoneally. Blood glucose levels were measured prior to injection and at various time points following injection. Blood glucose measurements were plotted against time and the area under the curve (AUC) was calculated using the positive incremental area method. With this method, only those areas that are above the baseline value are calculated and any value that falls below the baseline is ignored. Some groups have suggested concerns with this method because ignoring areas below the baseline is equivalent to discarding much of the variance in any readings below the baseline (101).

This should not pose a problem in these experiments because all of the blood glucose readings were above the baseline, except in some cases the last one or two readings at 180 and 240min were only very slightly below the baseline after blood glucose measurements leveled off back near the baseline value.

The hypothesis with these experiments was that the area under the GTT curve would increase as more beta cells are destroyed as the mice age causing an impairment in glucose metabolism. We began with a pilot experiment using BALB/c mice to determine if this method was feasible to use for a long term study in the DQ8α/DQ2β mice. GTTs were done on a group of BALB/c mice and then were repeated with the same mice two days later. As shown in Figure 4.4, the AUC fluctuated significantly from day to day in the mice. For example, a female BALB/c mouse 8 weeks of age had an AUC on Day 1 of 545.1 and on Day 3 this value was significantly lower at 38.76. We determined that GTTs would not be a useful method to study the change in glucose metabolism with age in the DQ8α/DQ2β mice and decided not to pursue this experiment any further at the time.

We used Western blotting to determine if DQ8α/DQ2β mice have autoantibodies directed towards proteins of the pancreas and beta cells. Extracts of pancreas and beta-TC tet cells (a mouse beta cell line) were blotted and probed with pooled NOD serum or pooled DQ8α/DQ2β serum (Figure 4.5). The NOD serum detected three protein bands from the beta-TC tet extract. The two larger proteins were also detected in the lane containing the pancreas extract, however the smaller of the three proteins was not detected. The same three bands were also detected in both the pancreas and beta-TC tet extract probed with DQ8α/DQ2β serum in addition to a fourth band that was not seen

with the NOD serum. No bands were seen when the blots were probed with the secondary antibody only or pooled serum from 16 week old BALB/c mice (data not shown). Although this is a crude method to detect autoantibodies in the serum of these mice it does indicate that DQ8α/DQ2β mice have some autoantibodies in their serum targeted towards proteins found in beta cells and some of these may be similar to those found in NOD mice. It cannot be determined for certain from these Western blots whether the exact same proteins were detected with the DQ8α/DQ2β and NOD serum and further experiments using 2D-gels could help elucidate this further.

Dr. George Eisenbarth's group at the Barbara Davis Center have developed ninety-six well filtration plate microassays for detecting autoantibodies to the three most common diabetes-associated autoantigens which include insulin, GAD65 and IA-2. Serum was collected from DQ8α/DQ2β mice and NOD mice and the serum samples were sent to Liping Yu at the Barbara Davis Center to be tested for insulin autoantibodies using the Ninety-Six-Well Filtration Plate Micro-IAA Assay. Serum producing a result greater than 0.01 is considered positive for insulin autoantibodies. Insulin autoantibodies were not detected in the serum from any of the DQ8α/DQ2β mice (Table 4.4). At the time there was not enough serum left over from this cohort of mice to perform further assays, however autoantibodies in these mice can be explored further in the future by testing serum for GAD65 and IA-2 autoantibodies.

Table 4.1. Incidence of spontaneous diabetes in DQ8α/DQ2β mice

Nov 19, 2002	First diabetic mouse: male; hemizygous for DQ; 27 weeks of age
Apr 25, 2003	Second diabetic mouse: male; homozygous for DQ; 42 weeks of age
Jun 11, 2003	Third diabetic mouse: male; homozygous for DQ; 27 weeks of age
Aug 5, 2003	Fourth diabetic mouse: male; homozygous for DQ; 64 weeks of age 4 out of 36 mice that lived to ≥25 weeks of age developed diabetes to date
Aug 6, 2003 – Aug 31, 2004	No mice developed diabetes
Aug 31, 2004	4 out of over 100 mice that lived to ≥25 weeks of age developed diabetes to date

Mice with two consecutive blood glucose measurements of ≥11.1mmol/L or one measurement of >33.3mmol/L were termed diabetic.

Table 4.2. Incidence of diabetes following cyclophosphamide injection

Strain	Incidence[1]			
	Females		Males	
	6 weeks	16 weeks	6 weeks	16 weeks
NOD	ND	4/4	ND	3/8
DQ8α/DQ2β	0/5	0/5	0/5	0/5

[1]Number of diabetic mice/number of mice in cohort
ND = Not Done

Male and female mice 6 and 16 weeks of age were injected twice intraperitoneally with 350mg/kg body cyclophosphamide 7 days apart. Blood glucose levels were monitored for 2 weeks starting from the day of the first injection. Diabetes was defined as two consecutive blood glucose measurements of ≥11.1mmol/L.

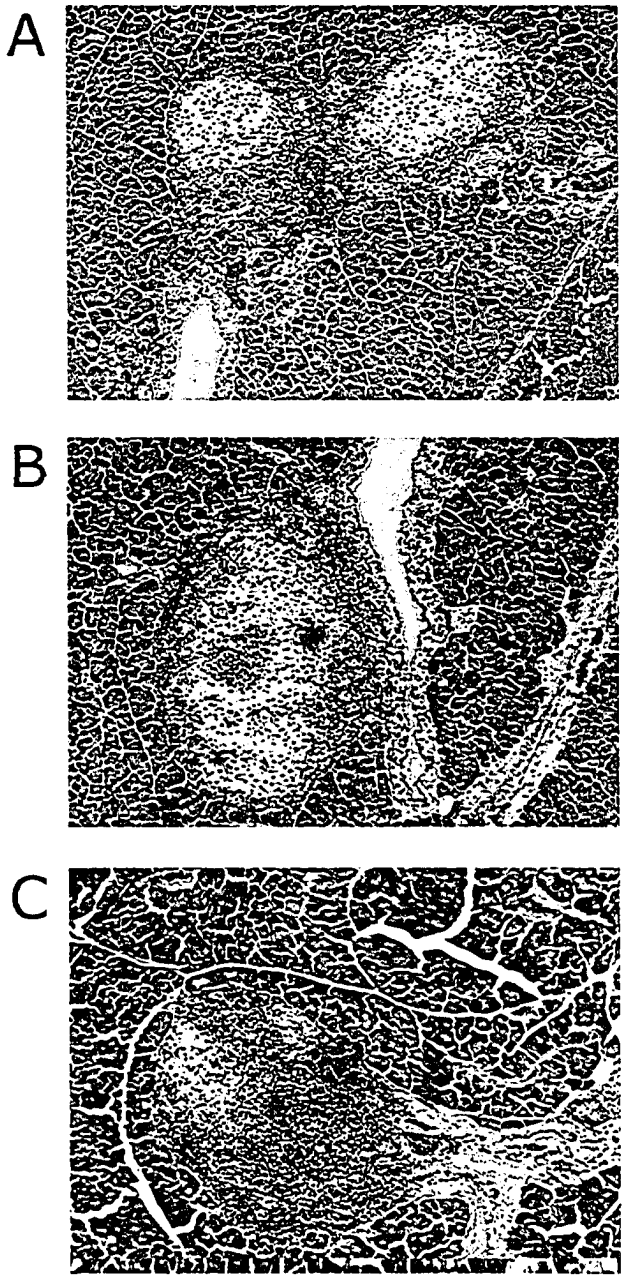


Figure 4.1. Histopathology of pancreas from DQ8α/DQ2β mice. Formalin-fixed, H&E stained pancreas sections were examined for islet infiltration. Representative sections showing peri-insulitis (**A**), moderate insulitis (**B**) and severe insulitis (**C**) are shown. Magnification x40.

Table 4.3. Histological assessment of insulitis

Strain	Sex	# mice in cohort	Age	Total islets counted	% islets of each grade		
					1	2	3
DQ8α/DQ2β	F	5	4-8	62	1.6	0	0
	M	5	4-8	75	1.3	0	0
	F	2	24	78	15.4	7.7	1.3
	M	2	24	79	6.3	12.7	3.8
	F	5	36	94	12.7	1.1	0
	M	5	36	80	2.5	0	0
DQ6	F	4	4-8	55	0	0	0
	M	4	4-8	30	0	0	0
	F	2	16-24	32	0	0	0
	M	2	16-24	43	0	0	0

Formalin-fixed, H&E stained pancreas sections from indicated mice were evaluated by the investigator blinded to the identity of the strain and scored for degree of insulitis. Individual islets were scored as follows: 1 = peri-insulitis, mononuclear cells surrounding the islet but no infiltration of islet architecture; 2 = moderate insulitis, less than 50% of the islet infiltrated; 3 = severe insulitis, greater than 50% of the islet infiltrated.

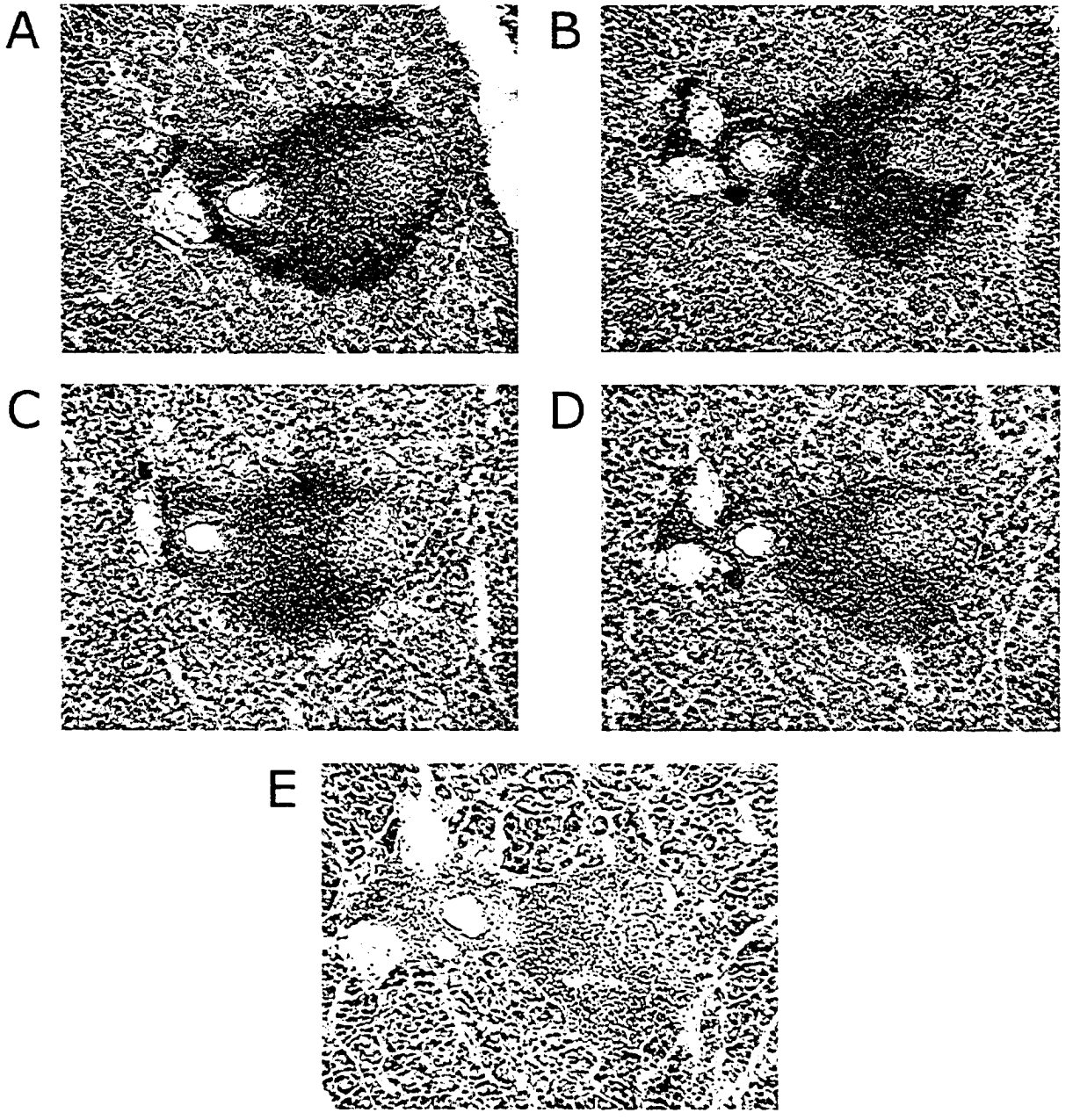
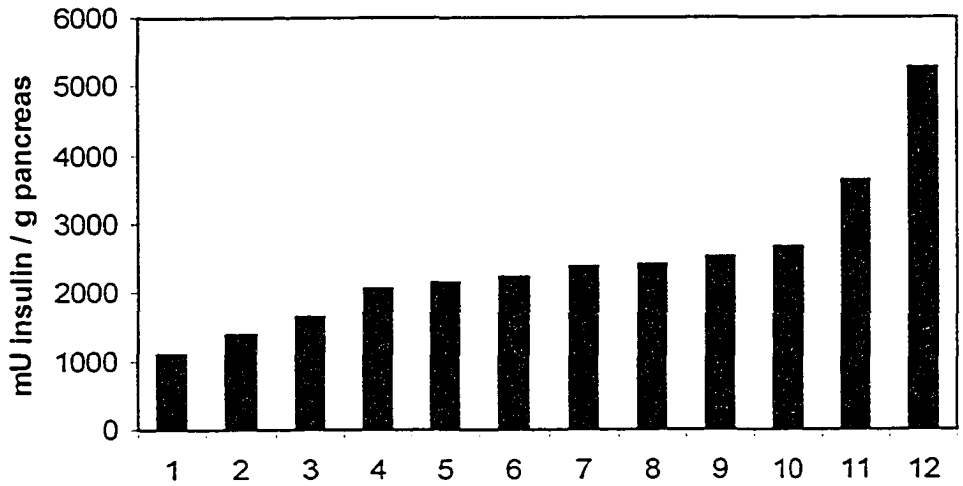


Figure 4.2. Characterization of immune cell subpopulations infiltrating the islets of DQ8α/DQ2β mice. Immunohistochemical staining of consecutive frozen pancreas sections shows a large number of B cells (**A**) and CD4+ T cells (**B**) and a smaller number of CD8+ T cells (**C**) infiltrating the islets. Very few macrophages (**D**) are present. Panel **E** shows an H&E stained section of the islet. Magnification x40.

A



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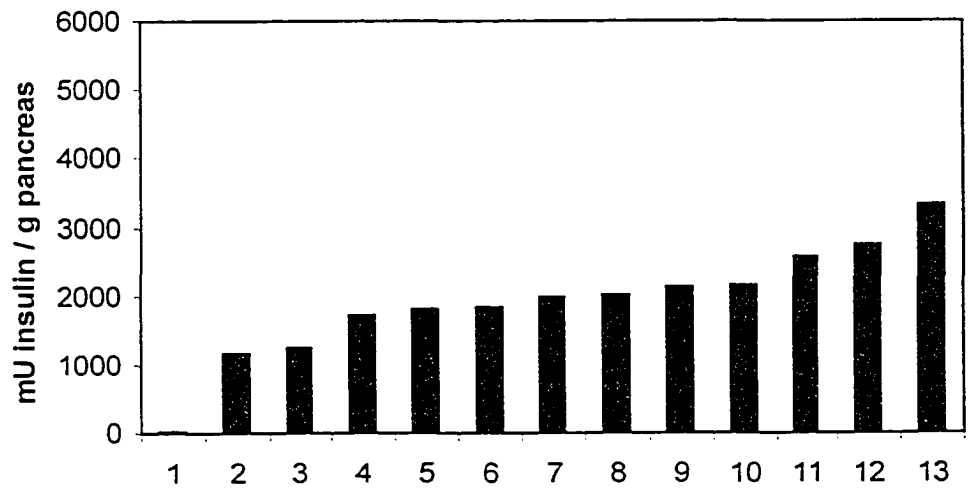


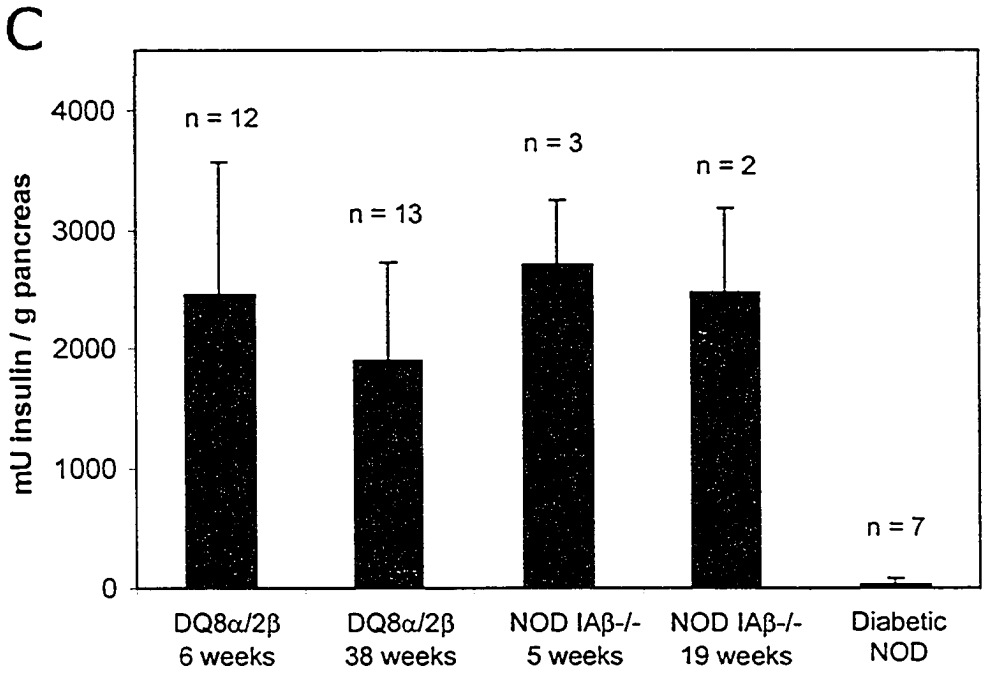
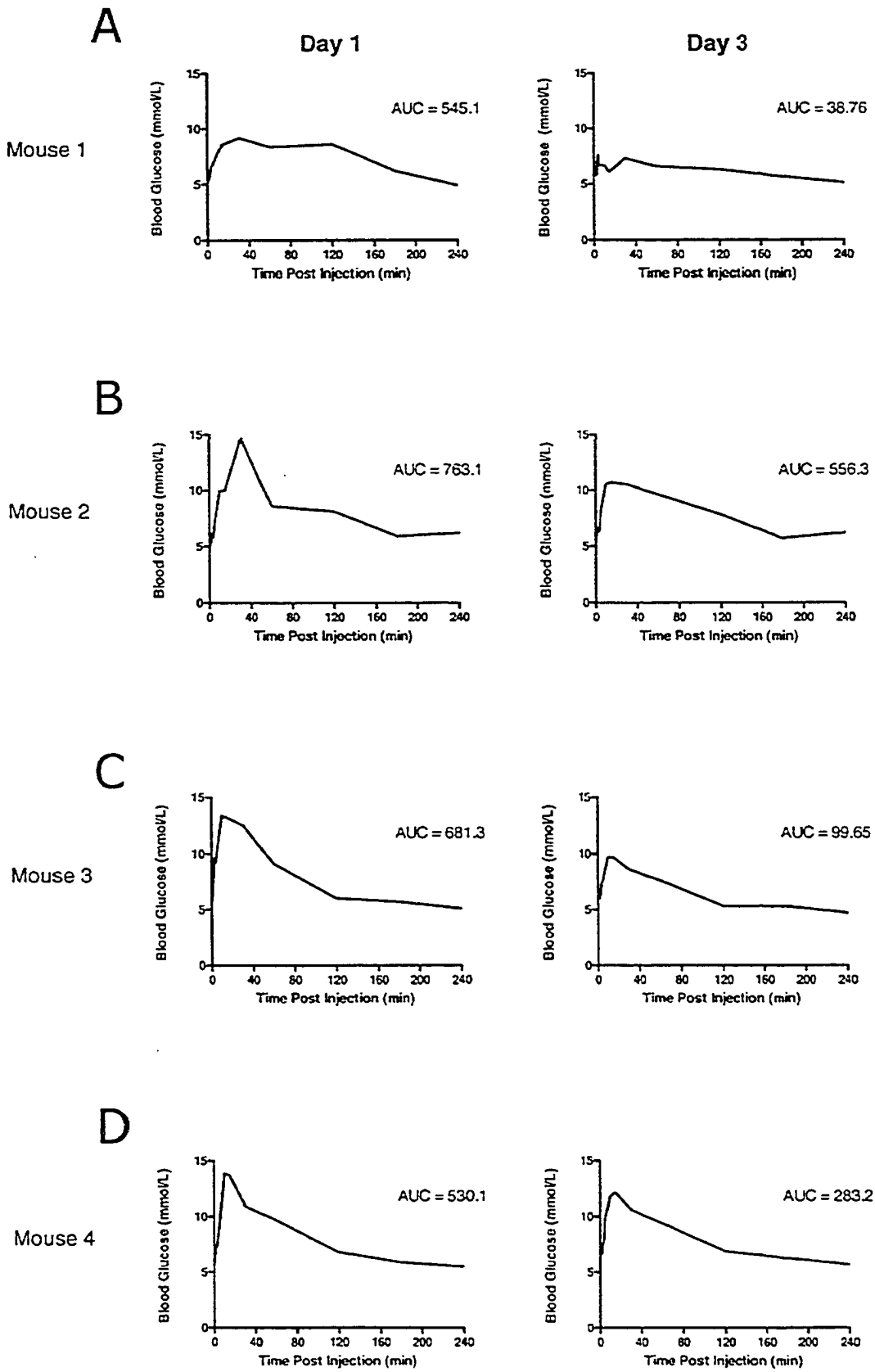


Figure 4.3. Determination of pancreatic insulin content. Pancreatic insulin content of individual 6 and 38 week old DQ8α/DQ2β mice are shown in panels **A** and **B** respectively. Average pancreatic insulin content of DQ8α/DQ2β, NOD IAβ null and diabetic NOD mice is shown in panel **C**.

Figure 4.4. Intraperitoneal glucose tolerance tests. Mice were fasted overnight and challenged intraperitoneally with glucose (2g/kg). Blood glucose levels were monitored prior to and at various times following injection (Day 1). This procedure was repeated with the same mice two days later (Day 3). BALB/c female mice 8 weeks (**A**) and 16 weeks (**B**) of age and male mice 8 weeks (**C**) and 16 weeks (**D**) of age were tested. Area under the curve (AUC) indicated for each graph was calculated using GraphPad Prism Software.



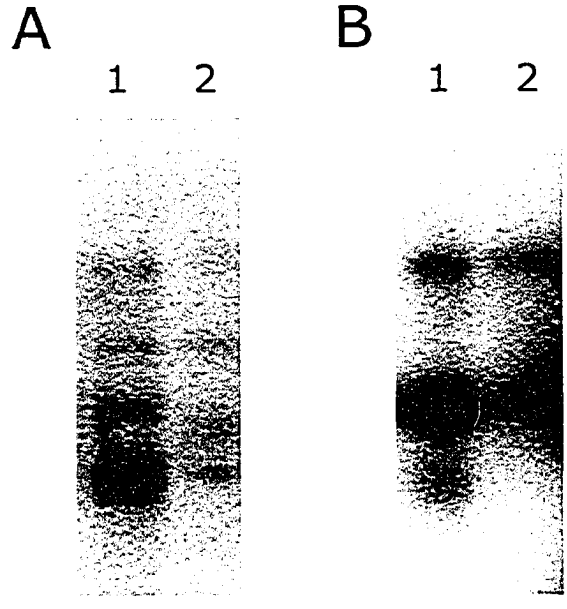


Figure 4.5. Detection of autoantibodies in the serum of DQ8α/DQ2β and NOD mice by Western blot. Extracts of β-TC tet cells (lane 1) and pancreas (lane 2) were probed with pooled serum from 36 week old DQ8α/DQ2β mice (**A**) and 25 week old NOD mice (**B**). Serum from 2 male and 2 female mice for each group was pooled. Two additional blots were probed as controls, one with BALB/c serum pooled from 2 male and 2 female mice 16 weeks old and another with secondary antibody only and showed no staining (data not shown).

Table 4.4. Mouse insulin autoantibodies

Strain	Age (weeks)	n	Mouse insulin autoantibody levels
DQ8α/DQ2β	4	7	-0.007 -0.010 -0.005 -0.007 -0.006 -0.002 -0.005
	8	4	0.000 -0.005 -0.011 -0.004
	16	7	-0.004 -0.007 -0.005 -0.009 -0.007 -0.004 -0.007
	24	3	-0.003 -0.003 -0.006
DQ8α/DQ2β (Diabetic)	42	1	-0.006
NOD	8	2	-0.004 0.001
	24	1	0.026

Serum samples from DQ8α/DQ2β and NOD mice were analyzed with the Ninety-Six Well Filtration Plate Micro-IAA Assay as described in the Materials and Methods. Values of >0.010 are considered positive for IAAs.

CHAPTER V

MAPPING OF DIABETES AUTOANTIGEN T CELL EPITOPES RECOGNIZED BY THE DQ8α/DQ2β TRANS-HETERODIMER

INTRODUCTION

Antigenic peptides are bound by MHC class II molecules in their peptide binding cleft which influences the selection and regulation of CD4+ T cells. The shape of the peptide binding cleft varies in different MHC class II molecules and plays an important role in determining which antigenic peptides can be bound (1). Some of these peptides may include self-peptides that can lead to the activation of autoreactive T cells which may contribute to the development of type 1 diabetes. Some groups have suggested that the unique structural features of the NOD MHC class II molecule, IAg7, may allow it to bind certain peptides that cannot be bound by other MHC class II molecules and give rise to high numbers of autoreactive CD4+ T cells (63,102,103). Structural studies of the diabetes-associated DQ8 molecule in humans and the IAg7 molecule in NOD mice have demonstrated significant similarities between the two molecules, particularly in the peptide binding pocket (104,105). The peptide binding grooves of both DQ8 and IAg7 are substantially wider around the non-aspartic acid residue located at position 57 of the beta chain. This unique structural feature may influence the peptide binding preferences of these molecules compared to other MHC class II molecules (106-108). DQ2β also has a non-Asp57 residue.

Human proinsulin, GAD65 and IA-2 have been identified as autoantigens associated with diabetes because of the high frequency of autoantibodies to these molecules in pre-diabetic patients and those with recent onset diabetes (97). To identify peptides from these diabetes autoantigens that can be presented by the DQ8α/DQ2β trans-heterodimer, we have studied the T cell responses to these proteins in the DQ8α/DQ2β transgenic mouse model. Results from our epitope mapping studies, as well as results from superantigen studies done by Sarah Wallbank in the Elliott Lab indicate that with respect to function and peptide binding properties, the IAg7 molecule may be more similar to the DQ8α/DQ2β trans-heterodimer than it is to DQ8.

RESULTS AND DISCUSSION

The first autoantigen that we examined was human GAD65. Prior to mapping the epitopes for the protein, we wanted to determine whether DQ8α/DQ2β mice could show GAD65 specific recall responses following immunization with the protein. Splenocytes were purified from DQ8α/DQ2β mice that had been immunized in the belly with 100μg of GAD65 and recall responses were measured in a ^{3}H-thymidine uptake assay. Very large proliferative responses to GAD65 were observed 7 days following immunization with the protein (Figure 5.1A). Interestingly, similar proliferative responses were seen in mice that had not been immunized with the protein (Figure 5.1B). Since the GAD65 that we used was produced by recombinant technology in *E. coli*, we hypothesized that these large proliferative responses may be attributed to contamination of the protein with endotoxin from the *E. coli*. Endotoxin, also known as LPS, is a component of the outer membrane of gram negative bacteria and is a potent mitogen of B cells that induces high

levels of proliferation, especially in mice (1). Endotoxin tests performed on all of the recombinant proteins made in the Elliott Lab that were used for these experiments revealed that GAD65 contained a large concentration of endotoxin (Table 5.1). The large amount of endotoxin present in the GAD65 was most likely responsible for the proliferation that was observed. The other three proteins that were tested which included GAD65 recdel, human proinsulin and IA-2 all contained very little endotoxin. GAD65 is expressed at extremely low levels in *E. coli* and is therefore a very difficult protein to make in large quantities and it is also difficult to make free of endotoxin. However, when a deleted form of GAD65 missing the 89 N-terminal amino acids is made it can be expressed easily and at much higher levels and also purified away from endotoxin (J.F. Elliott, communication). Therefore, we used this truncated version of GAD65, called GAD65 recdel for some of the subsequent experiments. We immunized DQ8α/DQ2β mice in the belly with 100μg of GAD65 recdel and measured recall responses to the protein. Splenocytes from immunized mice (Figure 5.2, columns 1 and 2) showed slightly higher levels of proliferation compared to a non-immunized mouse (Figure 5.2, column 5) indicating that a recall response to GAD65 recdel could be obtained. In an attempt to increase the recall response, we tried immunizing the mice by a different method. In this method, each mouse was immunized with a total of 100μg of GAD65 recdel in CFA in both hind foot pads, the base of the tail and the belly and were boosted 7 days later in the same manner as the initial immunization except the protein was emulsified in IFA. This method of immunization (Figure 5.2, columns 3 and 4) did not increase the amount of lymphocyte proliferation compared to the original immunization method.

After establishing that these mice could show recall responses to the protein, we then went on to delineate the fine specificity of the T cell responses to GAD65 that is restricted to the DQ8α/DQ2β trans-heterodimer. DQ8α/DQ2β mice were immunized with full length GAD65 and T cell proliferative responses were measured for each peptide from an overlapping set of synthetic peptides encompassing the full length of the GAD65 sequence (Table 5.2). Mapping of GAD65 led to the identification of four different immunodominant peptides that gave significant responses (Figure 5.3). Dominant proliferative responses were seen to peptides 9, 11, 21 and 28 giving SI values of 2.57±0.96, 3.66±0.29, 3.46±0.99 and 2.74±0.25 respectively.

Since splenocytes from the DQ8α/DQ2β mice showed significant levels of proliferation to GAD65 recdel even in the absence of prior immunization (ie. SI value of 3.77±0.17 with 20μg/mL GAD65 recdel, Figure 5.2), we were interested to see if splenocytes from non-immunized DQ8α/DQ2β mice would proliferate in response to any of the four immunodominant epitopes we identified. As shown in Figure 5.4A, splenocytes from non-immunized DQ8α/DQ2β mice proliferated in response to peptides 11 and 21, but not to peptides 9 and 28. Interestingly, splenocytes from non-immunized NOD mice also proliferated in response to these same two peptides (Figure 5.4B). All of these peptides were synthesized synthetically and were made free of endotoxin, so it is not likely that the responses seen were due to endotoxin contamination of the peptides.

To confirm the GAD65 epitopes that we identified for the DQ8α/DQ2β trans-heterodimer, we purchased several newly synthesized GAD65 peptides including peptides 9, 11 and 28 from the NAPS Unit Peptide Synthesis Laboratory, University of British Columbia. Additional versions of peptide 21 had previously been synthesized in

the Elliott Lab as a 20 amino acid version (P20) and a 14 amino acid version (P14) and were available for our experiments. T cell proliferation experiments were repeated with these new peptides and with other control peptides using splenocytes from GAD65 immunized and non-immunized DQ8α/DQ2β mice (Figure 5.5). Splenocytes from all of the mice tested failed to respond significantly to any of the newly synthesized GAD65 peptides compared to responses seen with the original set of peptides. These results were disappointing, however this could stem from the change in phenotype and lower DQ expression in the colony of DQ8α/DQ2β mice as mentioned previously in Chapter IV. The GAD65 experiments that were performed using the first set of overlapping peptides were completed between December 2002 and April 2003 during the period of time when a number of the mice developed diabetes. The experiments using the newly synthesized GAD65 peptides were done much later in August 2004, a year after the last DQ8α/DQ2β mouse developed diabetes. The change in phenotype and lower DQ expression may have resulted in the different levels of responses that were observed to the GAD65 peptides. Once a new DQ8α/DQ2β founder line is established and characterized (an effort currently underway in the Elliott Lab), it will be interesting to determine the GAD65 responses in the second founder line and compare them to the responses seen with the first DQ8α/DQ2β line.

Table 5.3, shows the DQ8α/DQ2β trans-heterodimer GAD65 epitopes we identified with the first set of synthetic peptides compared with published data for GAD65 epitopes identified for IAg7 and DQ8. Peptide 21 (201-220) has been identified as an immunodominant epitope for all three of the MHC class II molecules by all groups except Kaufman *et al*, which was one of the groups that first studied epitopes for IAg7.

Peptide 11 (101-120) has been identified as a dominant epitope for the DQ8α/DQ2β trans-heterodimer and at least sometimes for the DQ8 molecule, but not for IAg7. Peptide 9 (81-100) has been identified as a dominant eptiope for IAg7 and the DQ8α/DQ2β trans-heterodimer but not for DQ8. Peptide 28 (271-290), identified as a GAD65 epitope in the DQ8α/DQ2β mice appears to be unique to the trans-heterodimer. According to the pooled published data, DQ8 shares only one GAD65 epitope with IAg7, whereas we found that the DQ8α/DQ2β trans-heterodimer shares two epitopes with the NOD MHC class II molecule. This provides some evidence that the DQ8α/DQ2β trans-heterodimer may be more similar to the diabetogenic mouse IAg7 in structure and peptide binding properties than DQ8. Results from an experiment comparing SEB and TSST-1 superantigen responses in DQ8, DQ8α/DQ2β, DQ6 and NOD mice conducted by Sarah Wallbank in the Elliott Lab adds support to this hypothesis.

Superantigens are molecules that stimulate T cells displaying a particular beta chain variable region of the T cell receptor independent of antigen. They circumvent the normal mechanism for the activation of T cells by binding directly to MHC class II and the TCR. This trimolecular complex activates a large fraction of T cells and causes the release of massive amounts of inflammatory cytokines such as IL-2, TNF-α and IFN-γ (1). As shown in Figure 5.6, splenocytes from both DQ8α/DQ2β and NOD mice fail to respond to SEB in a ^{3}H-thymidine uptake assay, whereas splenocytes from DQ8 and DQ6 mice respond well. DQ8α/DQ2β and NOD splenocytes do however show proliferative responses to TSST-1, although these responses are not quite as high as those of DQ6 and DQ8 splenocytes. The similarity in GAD65 epitopes and superantigen

responses of the DQ8α/DQ2β trans-heterodimer and IAg7 gives some insight into the structural similarity of these two MHC class II molecules.

We also investigated T cell responses of DQ8α/DQ2β mice to human proinsulin and IA-2 in a similar manner to GAD65. We again began by determining if the DQ8α/DQ2β mice could show antigen specific recall responses to these proteins. We initially tried using the same method of immunization as we did for GAD65, in which mice were immunized once in the belly with 100μg of protein, however very poor recall responses were observed for both human proinsulin and IA-2 (data not shown). We repeated the experiment and tried to increase the responses by boosting the mice twice at 14 and 28 days following the initial inoculation. Boosting the mice did not increase the recall responses to either human proinsulin or IA-2. The proliferation that was observed for both proteins was very low and was similar to proliferation of splenocytes from non-immunized mice (Figures 5.7 and 5.8).

Despite the poor recall responses, we performed the epitope mapping experiments using overlapping sets of peptides for human proinsulin (Table 5.4) and IA-2 (Table 5.5) to see if we could identify any immunodominant epitopes. As shown in Figures 5.9 and 5.10, splenocytes from human proinsulin or IA-2 immunized DQ8α/DQ2β mice did not respond significantly to any of the peptides (ie. all SI values <2) and therefore no immunodominant epitopes for these proteins were identified for the DQ8α/DQ2β trans-heterodimer. This was not surprising since we could not establish good recall responses to either of the proteins. It should be noted that these experiments were performed during the same period of time as the original GAD65 experiments (ie. between December 2002 and April 2003 during the period of time when a number of the mice developed diabetes).

The lack of dominant human proinsulin epitopes for the DQ8α/DQ2β trans-heterodimer is similar to results obtained for the DQ8 molecule (Elliott Lab, unpublished results) in which no immunodominant epitopes were identified, although proinsulin peptides 5 and 24 also gave a hint of positivity for DQ8 as they do here for the DQ8α/DQ2β trans-heterodimer. Dr. Chella David's group mapped human pre-proinsulin epitopes for the DQ8 molecule using a similar method and found two peptides encompassing amino acids 1-24 and 44-63 to be immunodominant for the DQ8 molecule. Amino acids 1-24 of human pre-proinsulin are not contained within the proinsulin sequence and was therefore not studied here. The peptide sequence of pre-proinsulin encompassing amino acids 44-63 is partially found in a number of peptides used in this study, however we used different lengths of peptides than were used in David's study so no single proinsulin peptide of ours contains the entire 44-63 sequence (109). David's group has also mapped DQ8 epitopes for the IA-2 cytoplasmic region and found 6 immunodominant peptides with amino acid sequences encompassing residues 601-618, 616-633, 661-678, 721-738, 826-843 and 961-979 (110). Of note is the fact that in these experiments, David's group initially immunized the mice with mixtures of peptides, rather than with full length proteins and this may account for the different results they obtained.

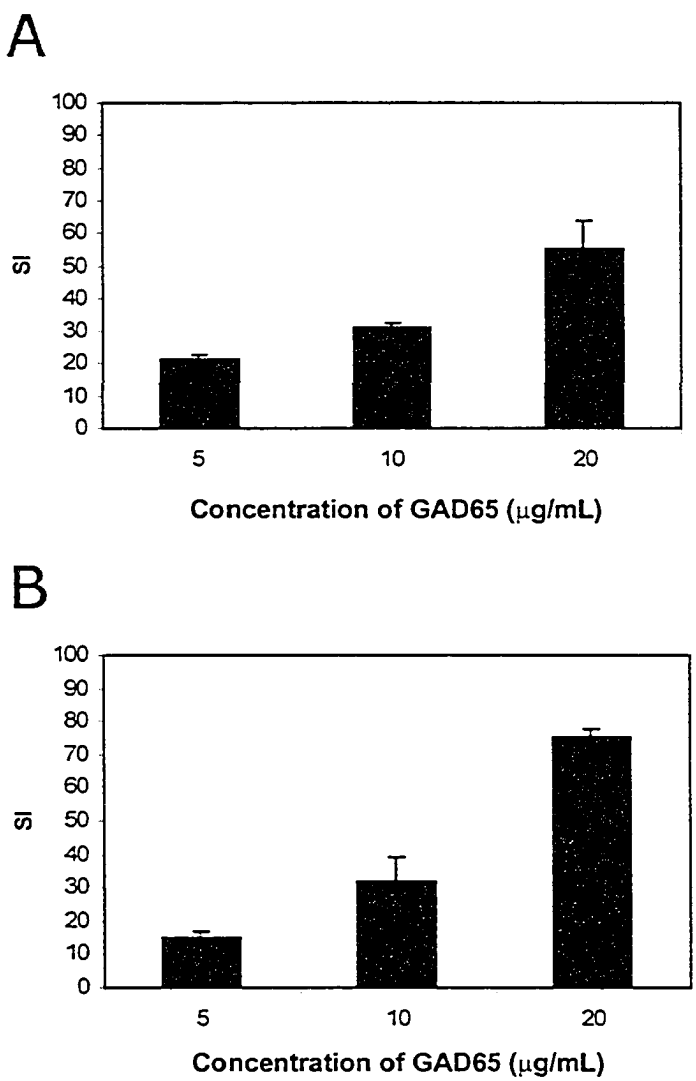


Figure 5.1. *In vitro* responses of splenocytes to GAD65. DQ8α/DQ2β mice were immunized in the belly with 100μg of GAD65 emulsified in CFA (**A**) or were not immunized (**B**). Splenocytes from 5 immunized mice were pooled and purified 7 days following immunization and splenocytes from 2 non-immunized mice were pooled and proliferation measured in a ^{3}H-thymidine uptake assay. Standard deviation of triplicate cultures are shown.

Table 5.1. Determination of endotoxin levels in proteins

Protein	EU/mg protein
GAD65	1100
GAD65 recdel	0.744
Human Proinsulin	0.646
IA-2	0.603

GAD65, GAD65 recdel, human proinsulin and IA-2 were tested for endotoxin using a BioWhittaker Limulus Amebocyte Lysate QCL-1000 kit.

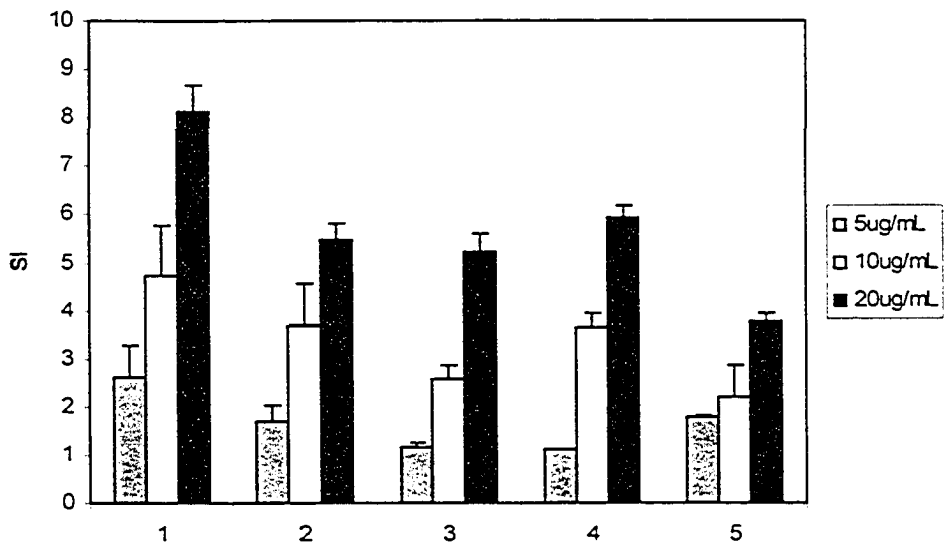


Figure 5.2. *In vitro* responses of splenocytes to GAD65 recdel. DQ8α/DQ2β mice were immunized once in the belly with 100μg of GAD65 recdel emulsified in CFA (1,2) or were immunized in the belly, base of the tail and both hind foot pads with 100μg of GAD65 recdel and boosted 7 days later in the same manner except using IFA (3,4) or were not immunized (5). Splenocytes (1,2,5) or splenocytes and popliteal lymph nodes (3,4) were purified 7 days following the final inoculation and proliferation was measured. Standard deviation of triplicate cultures are shown.

Table 5.2. Amino acid sequence of overlapping peptides of GAD65

Sequence	Position	#
MASPGSGFWSFGSEDGSGDS	1-20	1
FGSEDGSGDSENPGTARAWC	11-30	2
ENPGTARAWCQVAQKFTGGI	21-40	3
QVAQKFTGGIGNKLCALLYG	31-50	4
GNKLCALLYGDAEKPAESGG	41-60	5
DAEKPAESGGSQPPRAAARK	51-70	6
SQPPRAAARKAACACDQKPC	61-80	7
AACACDQKPCSCSKVDVNYA	71-90	8
SCSKVDVNYAFLHATDLLPA	81-100	9
FLHATDLLPACDGERPTLAF	91-110	10
CDGERPTLAF LQDVMNILLQ	101-120	11
LQDVMNILLQYVVKSFDRST	111-130	12
YVVKSFDRSTKVIDFHYPNE	121-140	13
KVIDFHYPNELLQEYNWELA	131-150	14
LLQEYNWELADQPQNLEEIL	141-160	15
DQPQNLEEILMHCQTTLKYA	151-170	16
MHCQTTLKYAIKTGHPRYFN	161-170	17
IKTGHPRYFNQLSTGLDMVG	171-190	18
QLSTGLDMVGLAADWLTSTA	181-200	19
LAADWLTSTANTNMFTYEIA	191-210	20
NTNMFTYEIAPVFVLLEYVT	201-220	21
PVFVLLEYVTLKKMREIIGW	211-230	22
LKKMREIIGWPGGSGDGIFS	221-240	23
PGGSGDGIFSPGGAISNMYA	231-250	24
PGGAISNMYAMMIARFKMFP	241-260	25
MMIARFKMFPEVKEKGMAAL	251-270	26
EVKEKGMAALPRLIAFTSEH	261-280	27
PRLIAFTSEHSHFSLKKGAA	271-290	28
SHFSLKKGAAALGIGTDSVI	281-300	29

Sequence	Position	#
ALGIGTDSVILIKCDERGKM	291-310	30
LIKCDERGKMIPSDLERRIL	301-320	31
IPSDLERRILEAKQKGFVPF	311-330	32
EAKQKGFVPFLVSATAGTTV	321-340	33
LVSATAGTTVYGAFDPLLAV	331-350	34
YGAFDPLLAV ADICKKYKIW	341-360	35
ADICKKYKIWMHVDAAWGGG	351-370	36
MHVDAAWGGGLLMSRKHKWK	361-380	37
LLMSRKHKWKLSGVERANSV	371-390	38
LSGVERANSVTWNPHKMMGV	381-400	39
TWNPHKMMGVPLQCSALLVR	391-410	40
PLQCSALLVREEGLMQNCNQ	401-420	41
EEGLMQNCNQMHASYLFQQD	411-430	42
MHASYLFQQDKHYDLSYDTG	421-440	43
KHYDLSYDTGDKALQCGRHV	431-450	44
DKALQCGRHVDVFKLWLMWR	441-460	45
DVFKLWLMWRAKGTTGFEAH	451-470	46
AKGTTGFEAHVDKCLELAEY	461-480	47
VDKCLELAEYLYNIIKNREG	471-490	48
LYNIIKNREGYEMVFDGKPQ	481-500	49
YEMVFDGKPQHTNVCFWYIP	491-510	50
HTNVCFWYIPPSLRTLEDNE	501-520	51
PSLRTLEDNEERMSRLSKVA	511-530	52
ERMSRLSKVAPVIKARMMEY	521-540	53
PVIKARMMEYGTTMVSYQPL	531-550	54
GTTMVSYQPLGDKVNFFRMV	541-560	55
GDKVNFFRMVISNPAATHQD	551-570	56
ISNPAATHQDIDFLIEEIER	561-580	57
ATHQDIDFLIEEIERLGQDL	566-585	58

Human GAD65 extends from residues 1-585. Peptides were synthesized as 20mers overlapping by 10 residues that span the entire human GAD65 sequence.

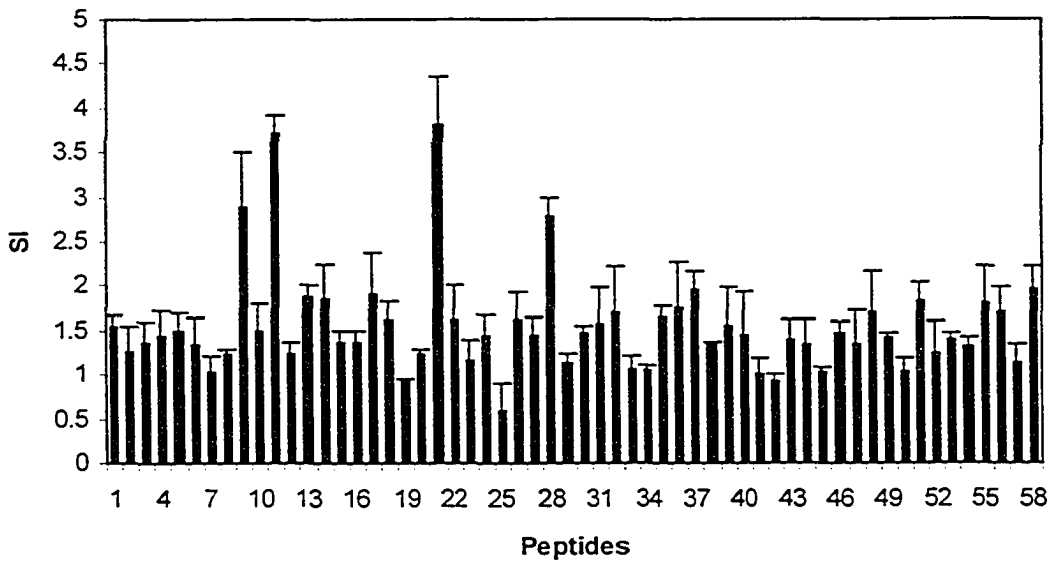


Figure 5.3. Determination of DQ8α/DQ2β-restricted T cell epitopes for GAD65 in DQ8α/DQ2β mice immunized with GAD65. DQ8α/DQ2β mice were immunized in the belly with 100μg GAD65 emulsified in CFA. Splenocytes from 5 immunized mice were purified and pooled 14 days following inoculation and tested against the GAD65 overlapping peptide set at peptide concentrations of 20μg/mL. Peptides are numbered consecutively from the N-terminus to the C-terminus. This experiment was repeated three times with triplicate cultures done for each experiment and the data is expressed as the mean SI values from all three experiments.

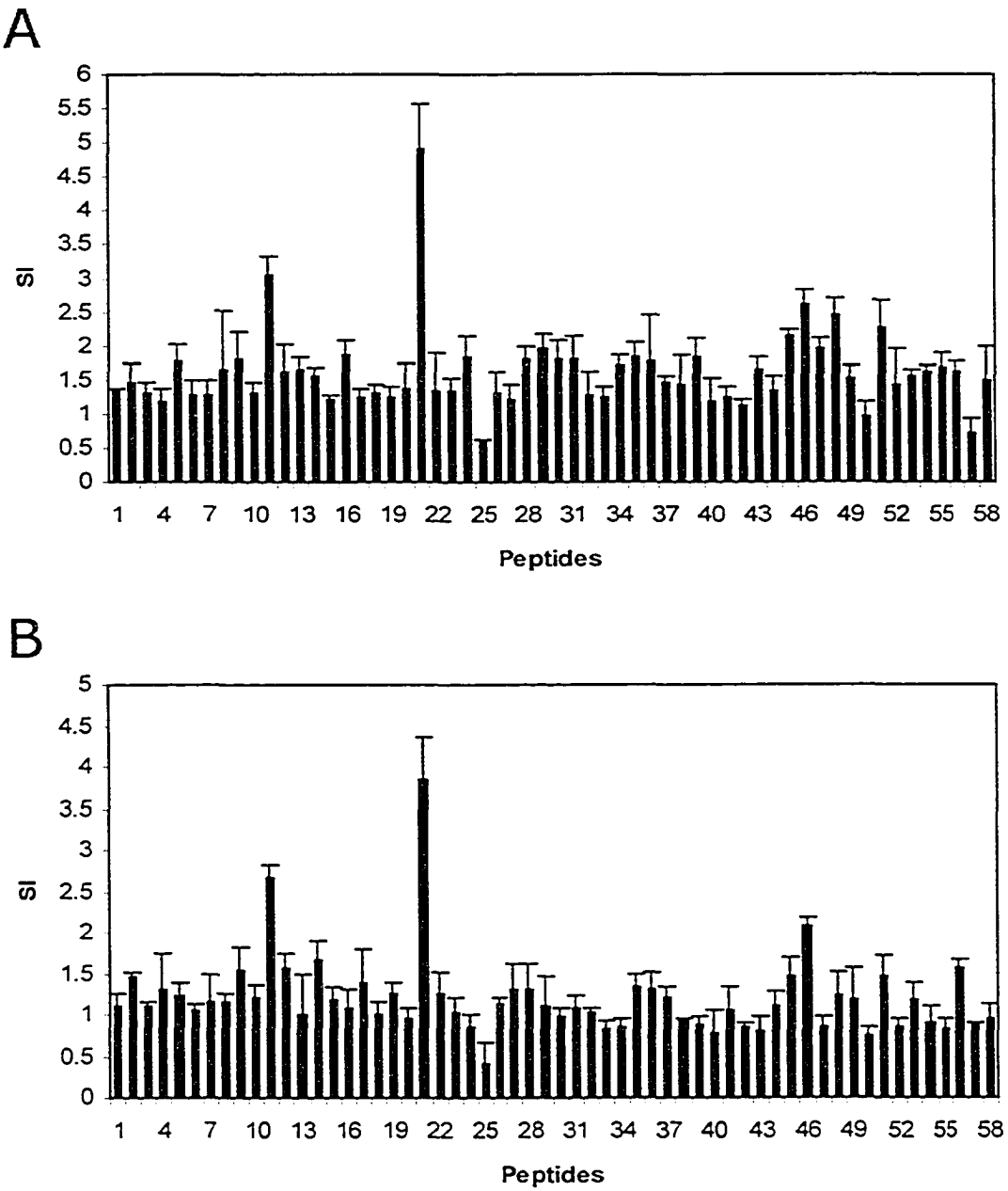


Figure 5.4. Determination of DQ8α/DQ2β-restricted T cell epitopes for GAD65 in non-immunized DQ8α/DQ2β (**A**) and NOD mice (**B**). Splenocytes from 5 non-immunized mice were purified and pooled for each experiment and tested against the GAD65 overlapping peptide set at peptide concentrations of 20μg/mL. Standard deviation of triplicate cultures are shown.

Figure 5.5. Attempted confirmation of identified DQ8α/DQ2β-restricted GAD65 epitopes with newly synthesized peptides. DQ8α/DQ2β mice were immunized in the belly with 100μg of GAD65 emulsified in CFA or were not immunized. Splenocytes were purified 7 days following inoculation and tested against GAD65 peptides 9, 11, 21 (P14 and P20 versions) and 28 as well as peptides from L18, Derp-1 and IA-2 as described in the Materials and Methods. (**A**) immunized male 8 weeks old, (**B**) immunized female 8 weeks old, (**C**) immunized male 30 weeks old, (**D**) immunized female 35 weeks old, (**E**) non-immunized male 30 weeks old, (**F**) non-immunized female 8 weeks old. Standard deviation of triplicate cultures are shown.

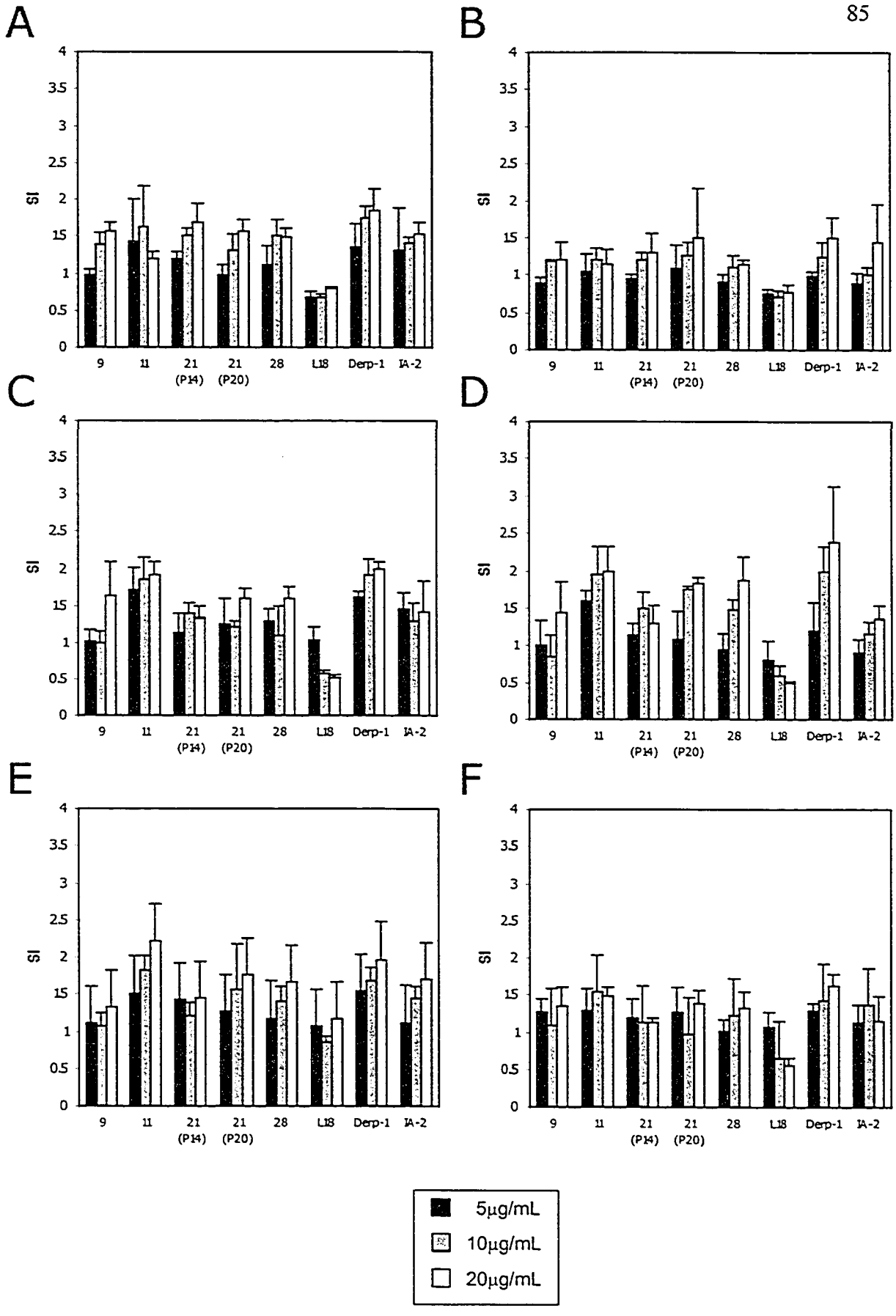


Table 5.3. Comparison of GAD65 epitopes identified in DQ8α/DQ2β, DQ8 and NOD mice.

DQ8α/DQ2β	DQ8[1]	DQ8[2]	NOD[3]	NOD[4]	NOD[5]
81-100				**78-97**	
101-120		**101-115**			
	121-140	126-140			
201-220	**201-220**	**206-220**	**206-220**	**202-221**	
			221-235	217-236	
	231-250				
					247-266
271-290					
			286-300		
		431-445			
		461-475			
	471-490				
					509-528
					524-543
		536-550			
			571-585		

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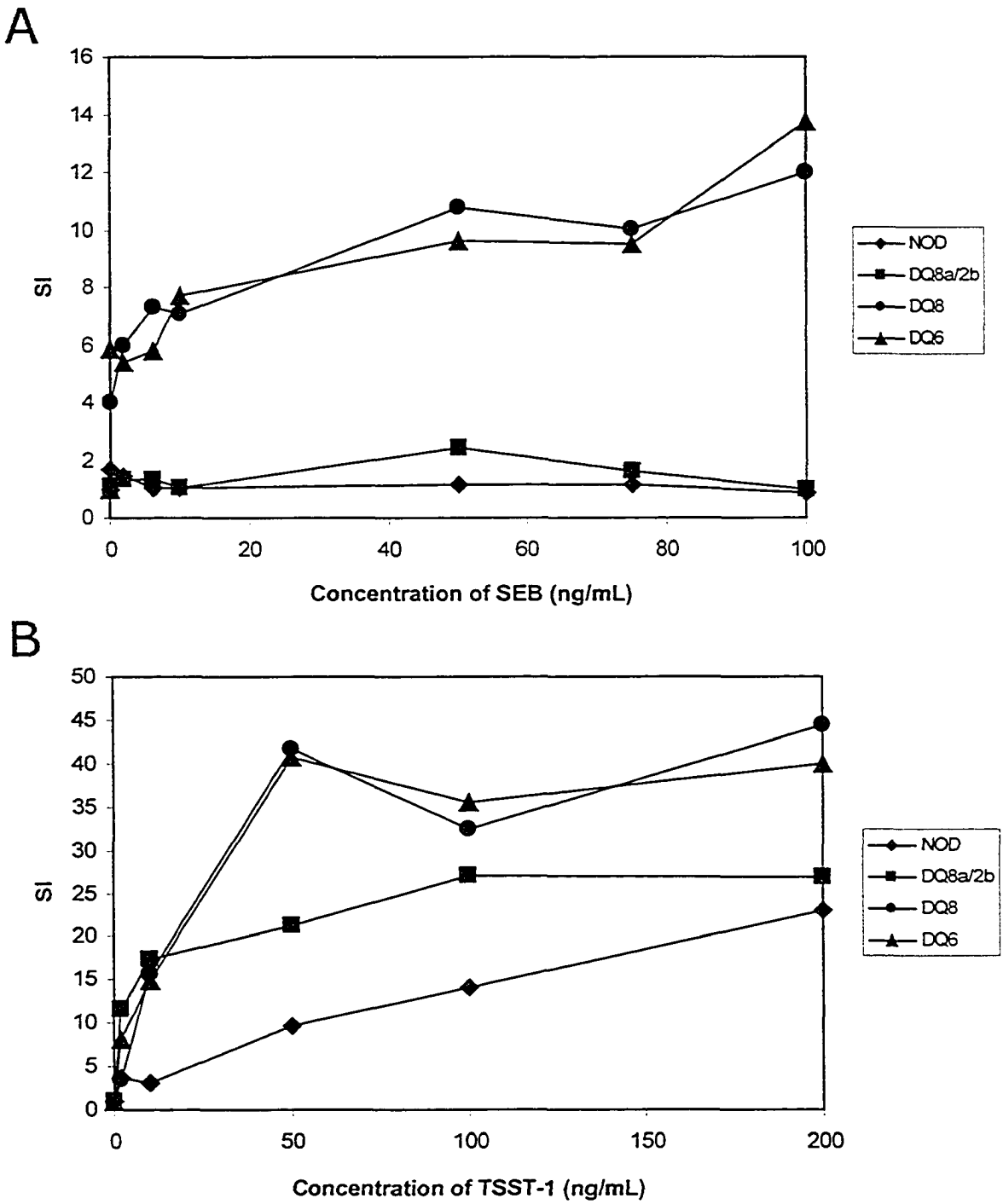

Figure 5.6. Proliferative responses of splenocytes from wildtype NOD, DQ8α/DQ2β, DQ8 and DQ6 mice to SEB and TSST-1 superantigens. Proliferation was measured in a ^{3}H-thymidine uptake assay. (Data courtesy of Sarah Wallbank).

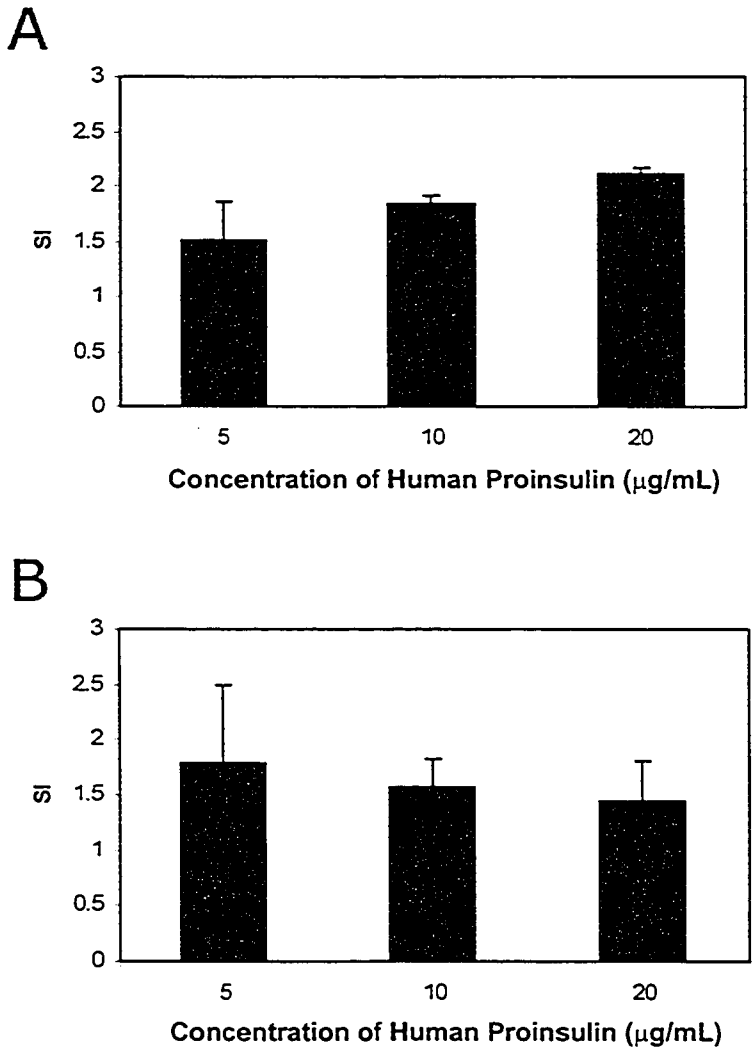

Figure 5.7. *In vitro* responses of splenocytes to human proinsulin. DQ8α/DQ2β mice were immunized in the belly with 100μg of human proinsulin emulsified in CFA and boosted 14 and 28 days later in the same manner except using IFA (**A**) or were not immunized (**B**). Splenocytes from 2 immunized mice were pooled and purified 7 days following final immunization and splenocytes from 2 non-immunized mice were pooled and proliferation measured in a ^{3}H-thymidine uptake assay. Standard deviation of triplicate cultures are shown.

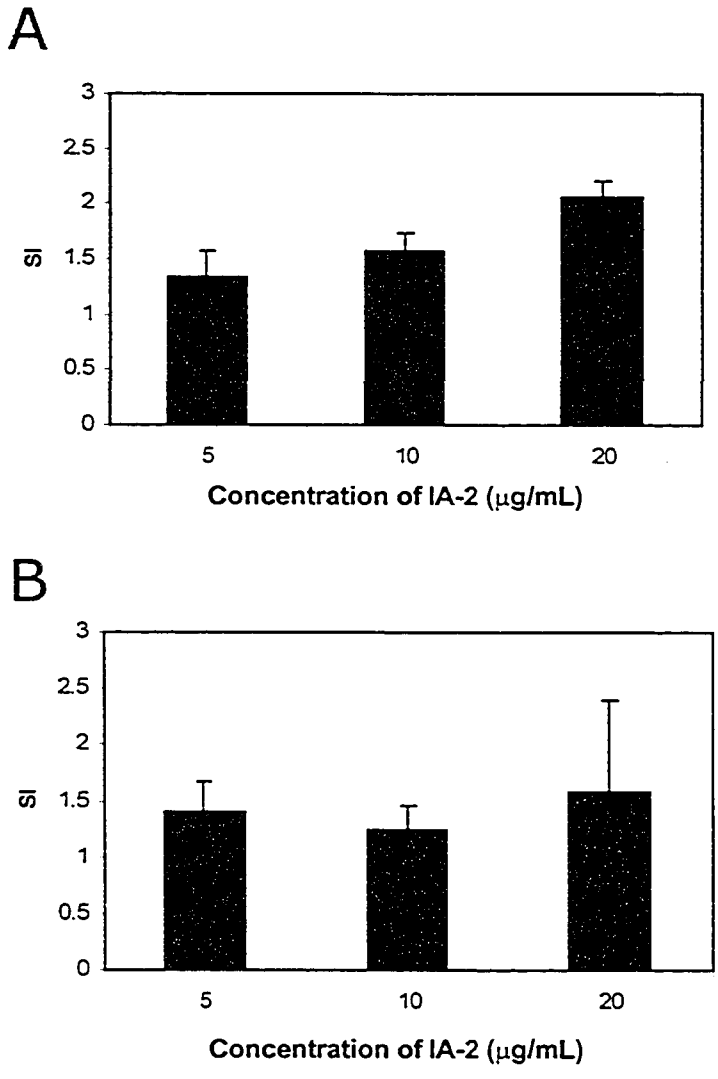

Figure 5.8. *In vitro* responses of splenocytes to IA-2. DQ8α/DQ2β mice were immunized in the belly with 100μg of IA-2 emulsified in CFA and boosted 14 and 28 days later in the same manner except using IFA (**A**) or were not immunized (**B**). Splenocytes from 6 immunized mice were pooled and purified 7 days following immunization and splenocytes from 2 non-immunized mice were pooled and proliferation measured in a ^{3}H-thymidine uptake assay. Standard deviation of triplicate cultures are shown.

Table 5.4. Amino acid sequence of overlapping peptides of human proinsulin

Sequence	Position	#
FVNQHLCGSHLVEAL	25-39	1
QHLCGSHLVEALYLV	28-42	2
CGSHLVEALYLVCGE	31-45	3
HLVEALYLVCGERGF	34-48	4
EALYLVCGERGFFYT	37-51	5
YLVCGERGFFYTPKT	40-54	6
CGERGFFYTPKTRRE	43-57	7
RGFFYTPKTRREAED	46-60	8
FYTPKTRREAEDLQV	49-63	9
PKTRREAEDLQVGQ	52-66	10
RREAEDLQVGQVELG	55-69	11
AEDLQVGQVELGGGP	58-72	12
LQVGQVELGGGPGAG	61-75	13
GQVELGGGPGAGSLQ	64-78	14
ELGGGPGAGSLQPLA	67-81	15
GGPGAGSLQPLALEG	70-84	16
GAGSLQPLALEGSLQ	73-87	17
SLQPLALEGSLQKRG	76-90	18
PLALEGSLQKRGIVE	79-93	19
LEGSLQKRGIVEQCC	82-96	20
SLQKRGIVEQCCTSI	85-99	21
KRGIVEQCCTSICSL	88-102	22
IVEQCCTSICSLYQL	91-105	23
QCCTSICSLYQLENY	94-108	24
CTSICSLYQLENYCN	97-110	25

Human proinsulin extends from residues 25-110 of pre-proinsulin. Peptides were synthesized as 15mers overlapping by 12 residues that span the entire human proinsulin sequence.

Table 5.5. Amino acid sequence of overlapping peptides of IA-2

Sequence	Position	#
SRQHARQQDKERLAA	600-614	1
RQQDKERLAALGPEG	605-619	2
ERLAALGPEGAHGDT	610-624	3
LGPEGAHGDTTFEYQ	615-629	4
AHGDTTFEYQDLCRQ	620-634	5
TFEYQDLCRQHMATK	625-639	6
DLCRQHMATKSLFNR	630-644	7
HMATKSLFNRAEGPP	635-649	8
SLFNRAEGPPEPSRV	640-654	9
AEGPPE PSRVSSVSS	645-659	10
EPSRVSSVSSQFSDA	650-664	11
SSVSSQFSDAAQASP	655-669	12
QFSDAAQASPSSHSS	660-674	13
AQASPSSHSSTPSWC	665-679	14
SSHSSTPSWCEEPAQ	670-684	15
TPSWCEEPAQANMDI	675-689	16
EEPAQANMDISTGHM	680-694	17
ANMDISTGHMILAYM	685-699	18
STGHMILAYMEDHLR	690-704	19
ILAYMEDHLRNRDRL	695-709	20
EDHLRNRDRLAKEWQ	700-714	21
NRDRLAKEWQALCAY	705-719	22
AKEWQALCAYQAEPN	710-724	23
ALCAYQAEPNTCATA	715-729	24
QAEPNTCATAQGEGN	720-734	25
TCATAQ GEGNIKKNR	725-739	26
QGEGNIKKNRHPDFL	730-744	27
IKKNRHPDFLPYDHA	735-749	28
HPDFLPYDHARIKLK	740-754	29
PYDHARIKLKVESSP	745-759	30
RIKLKVESSPS RSDY	750-764	31
VESSPSRSDYINASP	755-769	32
SRSDYINASPIIEHD	760-774	33
INASPIIEHDPRMPA	765-779	34
IIEHDPRMPAYIATQ	770-784	35
PRMPAYIATQGPLSH	775-789	36
YIATQGPLSHTIADF	780-794	37

Sequence	Position	#
GPLSHTIADFWQMVW	785-799	38
TIADFWQMVWESGCT	790-804	39
WQMVWESGCTVIVML	795-809	40
ESGCTVIVMLTPLVE	800-814	41
VIVMLTPLVEDGVKQ	805-819	42
TPLVEDGVKQCDRYW	810-824	43
DGVKQCDRYWPDEGA	815-829	44
CDRYWPDEGASLYHV	820-834	45
PDEGASLYHVYEVNL	825-839	46
SLYHVYEVNLVSEHI	830-844	47
YEVNLVSEHIWCEDF	835-849	48
VSEHIWCEDFLVRSF	840-854	49
WCEDFLVRSFYLKNV	845-859	50
LVRSFYLKNVQTQET	850-864	51
YLKNVQTQETRTLTQ	855-869	52
QTQETRTLTQFHFLS	860-874	53
RTLTQFHFLSWPAEG	865-879	54
FHFLSWPAEGTPAST	870-884	55
WPAEGTPASTRPLLD	875-889	56
PASTRPLLDFRRKV	880-894	57
RPLLDFRRKVNKCYR	885-899	58
FRRKVNKCYRGRSCP	890-904	59
NKCYRGRSCPIIVHC	895-909	60
GRSCPIIVHCSDGAG	900-914	61
IIVHCSDGAGRTGTY	905-919	62
SDGAGRTGTYILIDM	910-924	63
RTGTYILIDMVLNRM	915-929	64
ILIDMVLNRMAKGVK	920-934	65
VLNRMAKGVKEIDIA	925-939	66
AKGVKEIDIAATLEH	930-944	67
EIDIAATLEHVRDQR	935-949	68
ATLEHVRDQRPGLVR	940-954	69
VRDQRPGLVRSKDQF	945-959	70
PGLVRSKDQFEFALT	950-964	71
SKDQFEFALTAVAEE	955-969	72
EFALTAVAEEVNAIL	960-974	73
AVAEEVNAILKALPQ	965-979	74

The intracytoplasmic region of IA-2 extends from residues 601–979 of the full length protein. Peptides were synthesized as 15mers overlapping by 10 residues that span the entire intracytoplasmic region.

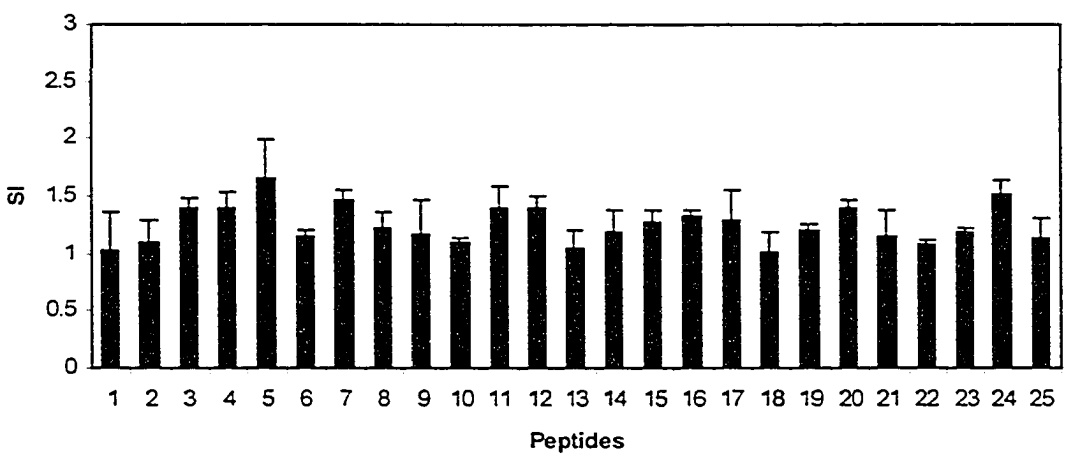

Figure 5.9. Determination of DQ8α/DQ2β-restricted T cell epitopes for human proinsulin. DQ8α/DQ2β mice were immunized in the belly with 100μg of human proinsulin emulsified in CFA. Mice were boosted 14 and 28 days later in the same manner except IFA was used. Splenocytes from 2 immunized mice were purified and pooled 7 days following the final inoculation and tested against the human proinsulin overlapping peptide set at peptide concentrations of 20μg/mL. Peptides are numbered consecutively from the N-terminus to the C-terminus. Standard deviation of triplicate cultures are shown.

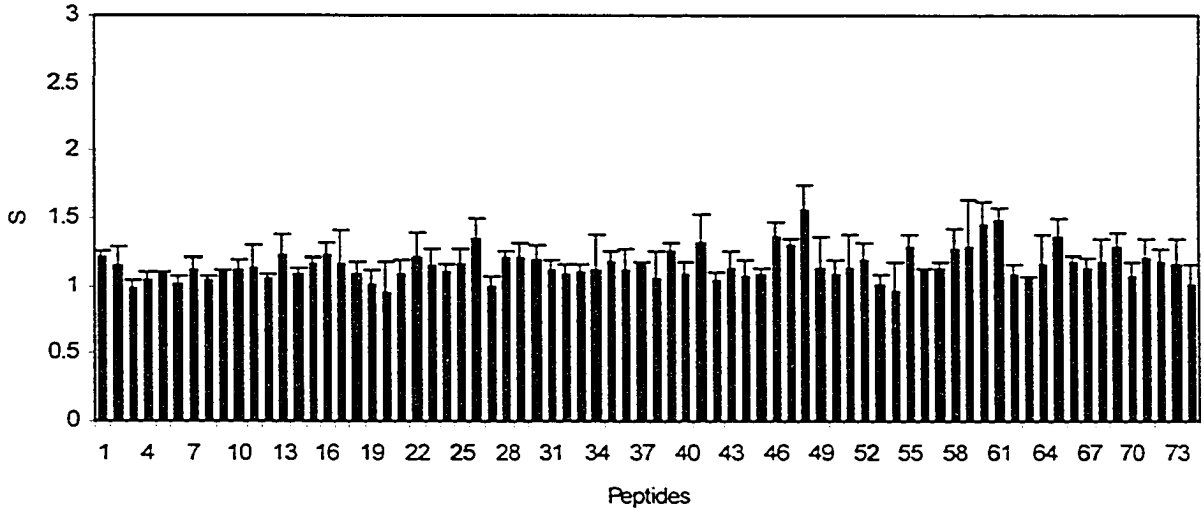

Figure 5.10. Determination of DQ8α/DQ2β-restricted T cell epitopes for IA-2. DQ8α/DQ2β mice were immunized in the belly with 100μg of IA-2 emulsified in CFA. Mice were boosted 14 and 28 days later in the same manner except IFA was used. Splenocytes from 6 immunized mice were purified and pooled 7 days following the final inoculation and tested against the IA-2 overlapping peptide set at peptide concentrations of 20μg/mL. Peptides are numbered consecutively from the N-terminus to the C-terminus. Standard deviation of triplicate cultures are shown.

CHAPTER VI

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this thesis was to create a humanized mouse model of type 1 diabetes and to specifically study the DQ8α/DQ2β trans-heterodimer that is associated with a high risk for diabetes development in humans. Certain HLA genes are associated with an increased risk for developing diabetes, but must act in concert with unknown environmental factors, rather than acting directly as simple mendelian disease genes. Therefore, HLA transgenic mice have not been predicted to spontaneously develop diabetes in the absence of additional disease factors due to the complex etiology and polygenic nature of the disease. HLA transgenic mice created by other groups expressing diabetes associated alleles in the absence of mouse class II have not spontaneously developed diabetes. The purpose of HLA transgenic mice has been to provide valuable tools to mimic the molecular interactions that give rise to the human disease and to identify HLA-restricted T cell epitopes for diabetes associated autoantigens.

Characterization of the DQ8α/DQ2β mice has shown that this is the first HLA-transgenic mouse model of diabetes that not only develops insulitis, but also spontaneously develops diabetes, at least in a few of the mice. A number of these mice developed diabetes during the first year immediately following establishment of the line, however after that period of time no additional mice developed the disease. We are currently in the process of creating a second founder line to confirm the initial results

discussed here. Assessment of the general immune function and composition of the DQ8α/DQ2β mice shows that the DQ8α/DQ2β molecule can effectively replace NOD IAg7. Numbers of subpopulations of immune cells vary slightly between NOD and DQ8α/DQ2β mice, however DQ8α/DQ2β mice and NOD mice show comparable recall immune responses to antigens such as OVA.

The absence of the NOD diabetogenic class I genes may be one reason for the low incidence of diabetes in the DQ8α/DQ2β mice. The class I genes of the DQ8α/DQ2β mice are K^b D^b from the 129 background instead of K^d D^b of NOD mice. The NOD IAβ null embryos that the DQ8α/DQ2β construct was microinjected into were originally created in the 129 background to make the IAβ deletion. These 129 IAβ null mice were then backcrossed onto NOD mice to create the NOD IAβ null line. During the backcrossing, an extended region of the 129 MHC locus was selected for which included the MHC class I genes leaving the NOD IAβ null mice with K^b D^b. It may be possible to increase the incidence of diabetes by adding diabetogenic class I to the DQ8α/DQ2β mice. One method of doing this would be to transgenically add K^d into these mice so that they will have the diabetogenic NOD class I molecule. Another method, that is currently underway in the Elliott Lab, is to cross the DQ8α/DQ2β mice with NOD HLA-A2 mice to add the diabetogenic human class I molecule HLA-A2 to these mice. NOD HLA-A2 mice (still having IAg7) develop diabetes much more rapidly than wildtype NOD mice.

An interesting step to take next with the DQ8α/DQ2β mice, would be to elucidate the roles that various immune cell subsets play in progression of the disease. One method of examining this would be to create DQ8α/DQ2β RAG-/- mice, lacking B cells and T

cells, that could be used to adoptively transfer immune cells from the DQ8α/DQ2β mice. Immune cell subpopulations from the DQ8α/DQ2β mice, such as CD4+ T cells and CD8+ T cells could be separately isolated by cell sorting and adoptively transferred into the DQ8α/DQ2β RAG-/- mice. The Elliott Lab has already performed similar experiments with respect to the DQ8 mice to determine which immune cells are important in the development of myocarditis in this animal model. With the DQ8 mouse model it was found that adoptively transferring an enriched population of CD4+ cells into DQ8 RAG-/- mice led to a more severe disease with a much greater degree of lymphocyte infiltration in the heart. If a similar result is seen upon adoptively transferring an enriched CD4+ T cell population into DQ8α/DQ2β RAG-/- mice, this could mean we would see a greater degree of insultis and beta cell destruction and therefore it is possible that a greater number of mice would develop diabetes.

Although the DQ8α/DQ2β mice lack IAβ they still have IAα, so one question that remains is whether IAα can dimerize with DQ2β and be co-expressed on the surface of APCs along with the DQ8α/DQ2β trans-heterodimer. One group that created DQ2/DR3 transgenic mice in the IAβ null background showed that IAα does not dimerize with DQ2β and DR3β in the mice (111). Dr. Chella David's group has made many DQ transgenic mice in the IAβ null background and they found that DQβ does not pair with IAα and only human class II molecules are expressed on the surface of cells (112). One method of assessing the MHC class II α and β chain pairing that the Elliott lab is currently exploring, is transfecting different combinations of DQ2, DQ8 and IA α

and β chains into cells and see which combinations can be expressed on the surface of the cells.

These HLA-transgenic mice enabled us to map T cell epitopes for diabetes associated autoantigens restricted to the DQ8α/DQ2β trans-heterodimer. We were able to obtain recall responses to purified human GAD65 recdel and subsequently identified four immunodominant GAD65 epitopes for the DQ8α/DQ2β trans-heterodimer. A comparison of published data with our data shows that the DQ8α/DQ2β trans-heterodimer shares more GAD65 epitopes with the NOD IAg7 molecule, than the DQ8 molecule does. In addition, DQ8α/DQ2β and NOD mice show similar responses to superantigens. DQ8α/DQ2β and NOD mice showed no response to SEB and both responded poorly to TSST-1 compared to DQ8 and DQ6 mice which responded well to both superantigens. Comparison of GAD65 epitopes and superantigen responses in DQ8α/DQ2β, DQ8 and NOD mice shows that the DQ8α/DQ2β trans-heterodimer and IAg7 may be more similar in function and peptide binding preferences than are DQ8 and IAg7. In contrast to experiments with GAD65, recall responses could not be obtained for human proinsulin and IA-2 in these mice and no DQ8α/DQ2β-restricted epitopes were identified for these two autoantigens. Interestingly, exactly the same result was obtained previously in the Elliott Lab using the DQ8 mice. It would appear that these two putative autoantigens are very poorly immunogenic, at least where only DQ8 or DQ8α/DQ2β molecules are available to present peptides to APCs.

The goal of this thesis to develop a humanized mouse model of type 1 diabetes and study the DQ8α/DQ2β trans-heterodimer has been accomplished, although this

research will be enhanced with the characterization of a second founder line that is being developed. Now that these mice have been characterized in general, the next logical direction to take this project would be to elucidate the molecular basis for the association of this particular HLA trans-heterodimer with susceptibility to type 1 diabetes. This could include performing structural studies for the trans-heterodimer and making comparisons to structural data available for the IAg7 and DQ8 molecules. We can define the peptide binding region of the trans-heterodimer and examine the peptide binding stability through binding studies, peptide elution, molecular modeling and crystallization of the peptide-trans-heterodimer complexes. Currently, the most widely used approach to identify epitopes is the method discussed in this thesis which involves utilizing sets of overlapping synthetic peptides spanning the entire sequence of the antigen of interest. One downfall of this approach is that it does not allow for the identification of naturally processed and presented epitopes. Dr. Mark Peakman's group developed a technique to identify naturally processed and presented epitopes by targeting native antigen onto APCs using a lectin-based antigen delivery system followed by elution of peptides and analysis of the peptides by mass spectrometry. This approach could be used to identify naturally processed peptides from autoantigens that can be presented by the DQ8α/DQ2β trans-heterodimer (113).

Vaccination with disease associated target antigens has resulted in significant decreases in disease severity in some animal models of experimental allergic encephalomyelitis, type 1 diabetes and antigen-induced arthritis. A number of groups have shown that administration of islet derived proteins or peptides of target autoantigens in the NOD mouse can cause a decrease in the incidence of diabetes or prevent diabetes

onset. Injection of insulin peptide B (9-23), nasal administration of insulin beta chain and intrathymic injection of baculovirus-derived GAD65 were all shown to decrease the incidence of diabetes during the period of time that they were administered (114-118). One group was able to prevent diabetes in mice treated with GAD65 peptides, however only two peptides from the collection of GAD65 epitopes were able to decrease the diabetes incidence at later ages providing evidence for the importance of identifying specific epitopes from target autoantigens (118-119).

HLA transgenic mice provide researchers with a way of studying the role of disease susceptible HLA molecules *in vivo* and they could facilitate the development of targeted immunotherapies. The DQ8α/DQ2β mice will provide a valuable tool to further our understanding of disease mechanisms involved in autoimmune diabetes and gain insights into the contribution of the DQ8α/DQ2β trans-heterodimer. Understanding the mechanisms of disease development will potentially enhance diagnosis and prediction of the disease in humans and could lead to development of peptide-based therapies. These peptide-based therapies could delay or prevent diabetes onset in pre-diabetic patients that have a susceptible genotype and have developed diabetes-associated autoantibodies.

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