uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Majid Mojibian

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Microbiology and Immunology)

GRADE / DEGREE

Department of Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Immune Response to Potential Diabetes-related Dietary Antigens, Including G1B1 in Patients with Type 1 Diabetes

TITRE DE LA THÈSE / TITLE OF THESIS

Fraser W. Scott

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Catherine Field

Mary-Ellen Harper

William Cameron

Ashok Kumar

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Immune Response to Potential Diabetes-related Dietary Antigens, Including Glb1 in Patients with Type 1 Diabetes

Majid Mojibian

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the PhD degree in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology
Faculty of Science
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-49382-3
Our file Notre référence
ISBN: 978-0-494-49382-3

ABSTRACT

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease dependent on gene-environment interaction. Food components are considered as environmental agents that influence the incidence of islet cell autoimmunity and T1D. Wheat proteins are harmless in healthy individuals, but in genetically susceptible individuals, wheat may be immunopathogenic. There are several reports that link wheat with increased risk of autoimmune diabetes. The major aim of this study was to determine whether there is an abnormal immune response to a mixture of wheat proteins (WP) or a purified wheat storage globulin homologue, Glb1 in T1D patients. If so, the question arises regarding the nature of the immune response and whether such reactivity is genetically controlled.

A sensitive CFSE-based proliferation assay and a capture ELISA technique were established to evaluate T cell immune response and Glb1 antibody level. Studies of young adults with T1D indicated that a subset of patients displayed abnormal humoral and cellular immune response to Glb1. T cell responses to WP and Glb1 were predominantly Th1 type and were associated with the diabetes risk gene HLA-DR4, and not HLA-DQ2, the main coeliac disease (CD) risk gene, suggesting the effect was diabetes-specific. Additional studies in children at high risk for diabetes with islet autoimmunity revealed that Glb1 antibody and zonulin were highly correlated, suggesting the presence of gut abnormalities related to tight junctions. In addition, increased immune response to Glb1 was present in children at high risk for diabetes who developed antibodies to the CD autoantigen, tTG. Interestingly, Glb1 antibodies appeared before tTG antibodies, suggesting that Glb1 antibody could have predictive value in people at risk of disease.

The data from this study clearly demonstrate for the first time the presence of abnormal humoral and cellular responses to wheat related dietary antigens in patients with T1D. Further, these data strongly suggest that the gut is not functioning normally and raises the possibility of a defect in the gut barrier, an immune imbalance and /or impaired oral tolerance.

DEDICATION

To all my family and friends

Especially

To my mother for her love

To Azarm, my best friend and my wife, I owe my deepest thanks to her whose love, patience and encouragement enabled me to complete this work.

To my wonderful sons, Aliereza and Amirreza for their love and patience

ACKNOWLEDGEMENT

It is my pleasure to thank many people who made this work possible.

- First and foremost I would like to thank my supervisor Professor Fraser Scott. I am deeply indebted to Dr. Scott whose support and encouragement helped me during the last three years and also I would like thank him for his patience.
- I especially wish to thank the members of the Scott laboratory past and present and others for their friendship, understanding and collaboration. This thesis would not have been possible without people who collaborated or shared their experiences with me, from the Scott lab: Drs. David Lefebvre, Habiba Chakir, Alexander Strom, Amanda MacFarlane, Lisa Kauri, Gen-Sheng Wang, Ms. Jennifer Crookshank and Ms. Brigitte Sonier. Dr. Reza Nokhbeh from the Dimock lab and Ms. Nancy Hampton at the Tissue Typing Laboratory at the Ottawa Hospital.
- I am grateful to Professor Lionel Filion for his guidance and support especially during the time when my former supervisor, Dr. Webb left the University of Ottawa.
- I would particularly like to express my appreciation to the volunteers who participated in this study.
- I wish to thank my former supervisor Dr John Webb and my thesis advisory committee members past, Professors Ashok Kumar, Lionel Filion, Subash Sad, Francisco Diaz-Mitoma and present, Professors Jacob Karsh, Majambu Mbikay, and Lionel Filion.
- I also thank Drs. Marian Rewers and Jill Norris at Denver, Colorado, Drs. Hubert Kolb and Markus Walz at the German Diabetes Center, Dusseldorf.
- I wish to thank the graduate academic assistants in the Biochemistry, Microbiology and Immunology Department, Ms. Nicole Trudel and Ms. Carol Ann Kelly.

Funding for this work was provided to Dr. Scott mainly by the Juvenile Diabetes Research Foundation (JDRF) and in part by the Canadian Institutes of Health Research (CIHR).

I am thankful to Canadian Blood Services for awarding me a doctoral fellowship and to the University of Ottawa for an admission scholarship and travel grants.

TABLE OF CONTENTS

ABSTRACT ..... **II**

DEDICATION ..... **IV**

ACKNOWLEDGEMENT ..... **V**

LIST OF BOXES, TABLES AND FIGURES ..... **IX**

LIST OF ABBREVIATIONS ..... **XI**

CHAPTER ONE **1**

LITERATURE REVIEW 1
- Brief history of diabetes 2
- Diabetes mellitus classification 2
- Type 1 diabetes (T1D) 4
  - Epidemiology 5
  - Etiology and Pathogenesis 5
  - Genetics 6
  - Environmental agents 8
  - Gut and its role in T1D 14
  - Immunology 15
- Coeliac disease and T1D 18

SUMMARY AND HYPOTHESIS 19

CHAPTER TWO **23**

MATERIALS AND METHODS 23
- Patients 24
- Sample preparation 26
- Antigens 27
- Flow cytometry 29
- CFSE-based flow cytometric proliferation assay 29
- Inhibition by polymyxin B 31
- Blocking HLA-DR 31
- Cytokine analyses 32
  - ELISA 32
  - Cytometric bead array 33
- Measurement of serum zonulin 34
- Measurement of anti-Glb1 antibody 34
- Measurement of LPS contamination in protein antigens 35
- HLA Typing 36
- Expression and purification of Glb1 37
- Statistical analyses 37

CHAPTER THREE **38**

T CELL RESPONSE TO WHEAT PROTEINS AND ASSOCIATION WITH HLA 38
Introduction 39
Experimental design and results 40
Results 41
T cell response to wheat protein in PBMC from T1D patients and controls 41
T cell response to dietary OVA and selected wheat antigens in a subset of T1D patients 42
IFN-γ and IL-6 are the dominant cytokines in supernatants of WP-stimulated PBMC 42
Immune reactivity against WP is not related to LPS contamination 52
Association of HLA-DR with immunity to wheat proteins 52
T cell responses to WP inhibited by monoclonal anti-DR IgG antibodies 53
Discussion 61

CHAPTER FOUR **67**

EXPRESSION AND PURIFICATION OF WP5212, A GLB1 HOMOLOGUE 67
Introduction 68
Experimental design and results 68
pBacPAK9-WP5212 transfer vector construction 69
Construction of a pBacPak6-His-Glb1 recombinant viral expression vector 70
Isolating recombinant virus and preparation of passage one virus stock 74
Preparation of passage two virus stock 74
Large scale His-Glb1 production in SF21 insect cells 75
Purification of His-tagged Glb1 from Baculovirus-infected SF21 cells 75
Discussion 76

CHAPTER FIVE **79**

IMMUNE REACTIVITY TO A GLB1 HOMOLOGUE IN A HIGHLY WHEAT-SENSITIVE PATIENT WITH TYPE 1 DIABETES AND COELIAC DISEASE 79
Introduction 80
Results 81
Subject 81
Antibody reactivity to Glb1 82
Clone WP5212 (Glb1) 82
Purified recombinant Glb1 protein 82
Reactivity of PBMC to WP components 82
Discussion 87

CHAPTER SIX **90**

IMMUNE REACTIVITY AGAINST GLB1 IN YOUNG ADULT T1D PATIENTS AND CHILDREN WITH T1D OR CD AUTOIMMUNITY 90
Introduction 91
Experimental design 91
Group 1 Young adult T1D patients 92
Results 92

T cell reactivity to Glb1 in PBMC from T1D patients and controls 92
T cell responses to Glb1 inhibited by monoclonal anti-DR antibodies 92
Immune response to Glb1 recombinant protein is not due to LPS contamination 93
Coincidence of $CD3^+$ T cell responses to WP and Glb1 in young adult T1D patients 93
IFN-γ and IL-6 are the dominant cytokines in the supernatant of Glb1 stimulated PBMC 93
Establishing a Glb1 ELISA assay using purified recombinant Glb1 94
Glb1 antibody levels increased in a subset of T1D patients 99
Coincidence of T cell and antibody responses to Glb1 in a subset of T1D patients ..99
Group 2 Children with T1D islet autoantibodies 103
Results 103
Group 3 Children with coeliac disease autoimmunity (tTG autoantibodies) 107
Results 108
Glb1 antibody level is higher at the time of seroconversion to tTG IgA 108
IgG Glb1 antibodies are increased before IgA-tTG in subjects with CD 108
Summary and Discussion 115
Study Group 1 Young adult T1D patients 115
Study Group 2 Children with islet autoimmunity (DAISY) 117
Study Group 3 Children at risk of developing coeliac disease (CEDAR) 119
Conclusion: 120

CHAPTER SEVEN 122

SUMMARY AND DISCUSSION 122
Potential role of wheat reactive immune cells in T1D pathogenesis 126
Model for involvement of dietary wheat proteins in development of T1D 128
Future directions 131
Conclusions 134

REFERENCES 136

STATEMENT OF CONTRIBUTION OF COLLABORATORS 154

COPYRIGHT PERMISSION 155

CURRICULUM VITAE 156

LIST OF BOXES, TABLES AND FIGURES

Box 1. 1 Classification of diabetes mellitus 3
Box 1. 2 Type 1 diabetes subtypes, preclinical and clinical 4
Box 1. 3 Genetic susceptibility to type 1 diabetes 7

Table 2.1 Description of subjects. 25
Table 3.1 Characteristics of individual subjects in the T1D study group in Ottawa 46
Table 3.2 Characteristics of individual subjects in the control study group in Ottawa 48
Table 3.3 LPS concentration in T cell antigens 55
Table 3.4 Frequency of MHC class II risk genes, HLA-DR and DQ in control subjects and T1D responders, non-responders 57
Table 6.1 Within assay precision for Glb1 assay 100
Table 6.2 Glb1 antibody level and age of children at risk of developing Coeliac Disease (CEDAR study) 111

Figure 3.1 Antigen-specific $CD3^+$ T cell proliferation 44
Figure 3.2 T cell responses to three different concentrations of wheat protein 45
Figure 3.3 T cell proliferation in response to wheat proteins and other antigens 49
Figure 3.4 Correlation between WP T cell response and age, duration of diabetes and T cell response to other antigens 50
Figure 3.5 Cytokines produced by WP-stimulated PBMC at day 8 of culture 51
Figure 3.6 Polymyxin B did not inhibit wheat protein induced T cell responses in T1D patients 56
Figure 3.7 Relation between wheat-induced T cell response and HLA diabetes risk alleles 58
Figure 3.8 HLA-DR antibodies block T1D T cell response to wheat proteins 59
Figure 3.9 Anti HLA-DR antibody blocks secretion of wheat-induced Th1 cytokines 60
Figure 4.1 Construction of BacPak9-His-Glb1 transfer vector and recombinant BacPak6 His-Glb1 baculovirus expression vector 71

Figure 4.2 Amplification, purification, and isolation of BacPak9 His-Glb1 transfer vector. 72
Figure 4.3 Expression and purification of recombinant His-Glb1 protein. 73
Figure 5.1 Wheat-sensitive patient with Glb1 antibody reactivity 84
Figure 5.2 Serum Glb1 antibodies in a T1D/CD patient and controls determined using ELISA 85
Figure 5.3 Wheat protein-induced T-cell reactivity in a T1D/CD patient but not in controls 86
Figure 6.1 T cell responses to Glb1 homologue in T1D patients blocked by ..... Anti HLA-DR..... 95
Figure 6.2 Polymyxin B did not inhibit Glb1 induced T cell response 96
Figure 6.3 Coincidence of $CD3^+$ T cell responses to WP and Glb1 in T1D patients 97
Figure 6.4 Cytokine concentrations in supernatants from Glb1-stimulated PBMC from T1D patients and controls 98
Figure 6.5 Serum Glb1 antibody level in patients and controls determined using ELISA .100
Figure 6.6 Coincidence of T cell and antibody response to Glb1 in individual T1D patients 102
Figure 6.7 Serum Glb1 antibodies among children with islet autoimmunity (DAISY Study) 105
Figure 6.8 Glb1 antibody levels before and after appearance of islet autoimmunity..... 106
Figure 6.9 Serum Glb1 antibodies in cases and controls analyzed using ELISA (CEDAR study) 110
Figure 6.10 Serum Glb1 antibodies in cases based on visit type (CEDAR study)..... 112
Figure 6.11 Comparison of Glb1 antibody level and IgA-tTG antibodies in coeliac disease autoimmunity (CDA) cases (CEDAR study). 113
Figure 6.12 Comparison of Glb1 antibody level and IgA-tTG antibodies in coeliac disease autoimmunity (CDA) cases..... 114
Figure 7. 1 A model for involvement of dietary wheat proteins in development of T1D ...133

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cell Cytotoxicity
APC	Antigen Presenting Cell
BBc Rat	BioBreeding control Rat
BBdp Rat	Diabetes-prone BioBreeding Rat
BSA	Bovine Serum Albumin
C3	Complement protein C3
CBV	Coxsackie B virus
CD	Coeliac Disease
CDI	Cell Division Index
CEDAR	**Co**Eliac **D**isease **A**utoimmunity **R**esearch
CFDA	5,6-carboxyfluorescein diacetate succinimidyl ester
CFSE	5,6-carboxyfluoresein succinimidyl ester
DAISY	**D**iabetes **A**utoimmunity study **i**n the **Y**oung
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAD	Glutamic Acid Decarboxylase
GALT	Gut Associated Lymphoid Tissue
Glb1	Homologue of the wheat storage globulin-1 protein
HC	Diabetes retardant AIN-93G hydrolyzed casein-based diet
HLA	Human Leukocyte Antigen
HRP	Horse-Radish Peroxidase
HSP	Heat Shock Protein
IA	Islet Autoimmunity
IA-2	Insulinoma Associated Autoantigen 2
IDDM	Insulin Dependent Diabetes Mellitus
IFN-γ	Interferon-γ
IgG	Immunogolobulin G

IL-	Interleukin
LF	Lethal Factor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Node
NADH	Reduced Nicotinamide Adenine Dinucleotide
Ni-NTA	Ni^{2+}-nitrilotriacetic acid
NOD	Non-Obese Diabetic mouse
OD	Optical Density
OVA	Chicken egg white ovalbumin protein
PBMC	Peripheral Blood Mononuclear Cell
PHA	Phytohemagglutinin
PLN	Pancreatic Lymph Nodes
PmB	Polymyxin-B
SDS-Page	Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis
SF21	Spodoptera frugiperda 21 insect cell
T1D	Type 1 diabetes
T1D/CD	Type 1 diabetes and Coeliac Disease
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
Th1	T helper 1
Th2	T helper 2
TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TRIGR	**T**rial to **R**educe **I**DDM in the **G**enetically at **R**isk study
TT	Tetanus Toxoid
tTG	tissue transglutaminase
WP	Wheat Proteins

Chapter One

Literature review

Brief history of diabetes

Aretaeus was the first who used the term 'diabetes' from the Greek word for a siphon. Matthew Dobson described hyperglycemia, which is an indication of diabetes, for the first time in 1776 "he found that the serum as well as the urine in a diabetic patient tasted sweet". John Rollo (1809) used the adjective 'mellitus' from the Greek and Latin words meaning 'honey' and called this disease Diabetes Mellitus [1]. In the late 19th century, the pancreas was discovered as the controlling organ for carbohydrate metabolism. Many attempts were made to isolate the effective secretion of pancreas. Finally, in 1921 Banting and his colleagues discovered insulin, an achievement for which they received the Nobel Prize jointly in 1923. Von Meyenburg in 1940 reported an inflammatory insulitis (lymphocytic infiltration of the islands of Langerhans) in diabetic patients, which later provided evidence of an autoimmune process in a subpopulation of diabetic patients [2,3]. Since the discovery of insulin, there have been no other major discoveries that significantly affected the lifespan of diabetic patients [1].

Diabetes mellitus classification

Diabetes mellitus is a metabolic disorder specifically affecting carbohydrate metabolism. It is a disease characterized by chronic hyperglycemia due to deficiency of insulin action caused by a loss of the physical, functional β-cell mass and/or decreased insulin sensitivity [4-6]. According to WHO classification and ADA recommendations, diabetes mellitus is divided into four major groups (Box 1.1).

Box 1. 1 Classification of diabetes mellitus†

I. Type 1 diabetes
  a) Immune-mediated
  b) Idiopathic (autoantibody negative)
II. Type 2 diabetes
III. Other specific types of diabetes
  a) Genetic defects of β-cell function (e.g., MODY)
  b) Genetic defects in insulin action
  c) Diseases of the exocrine pancreas
  d) Endocrinopathies
  e) Drug- or chemical-induced forms
  f) Infections
  g) Uncommon forms of immune-mediated diabetes (e.g., stiff man syndrome, anti-insulin receptor antibodies)
  h) Other genetic syndromes, sometimes associated with diabetes
IV. Gestational diabetes mellitus

† adapted from [7]

Type 1 diabetes (T1D)

The term Type 1 diabetes was adopted by the American Diabetes Association and the World Health Organization (1997 and 1999 respectively), and replaces the previous term Insulin-Dependent Diabetes Mellitus (IDDM) [8]. This form of diabetes is characterized by destruction of the insulin-producing β-cells. The new classification distinguishes two forms of type 1 diabetes: the autoimmune form, type 1a, and an idiopathic form, type 1b [6]. Box 1.2 shows the clinical subtypes of Type 1 diabetes [8].

Box 1. 2 Type 1 diabetes subtypes, preclinical and clinical†

Type 1 diabetes

Preclinical

- Autoantibodies present/OGTT normal
- Autoantibodies ++/OGTT abnormal
- Autoantibodies++/fasting hyperglycemia

Clinical type 1 diabetes (ketosis-prone)

- 1a: Autoantibodies present (autoimmune) ~95%
- 1b: Autoantibodies absent (idiopathic) ~5%
- Explosive onset (type 1c)
- Rapid onset
- Late onset (LADA): 10-20%

LADA, latent autoimmune diabetes in adults; OGTT, oral glucose tolerance test

† adapted from [8]

Epidemiology

Type 1 diabetes is one of the most common chronic diseases of children aged less than 16 and affects approximately 0.4% of the population of developed countries. Although T1D is known as a disease of childhood, recent epidemiological studies indicate that the incidence is comparable in adults 9. There is geographical variation in incidence of this disease. China has a low rate of about 0.57 cases per 100,000 of the population younger than 18 years of age per year. The UK has an intermediate incidence of 18-20 per 100,000/ year and very high rates of 48-49 per 100,000/ year are reported in Finland and Sardinia 10-13. The Avalon Peninsula of Newfoundland in Canada has a high rate of 36 per 100,000/year 14.

The incidence of diabetes has been increasing at a rate of 2-5% per year worldwide. Interestingly, in populations with lower incidence this increase is very rapid compared with the populations in high incidence countries such as Finland 15-17. Several publications during the 1990s reported an increasing trend in incidence of this disease 10. In addition, in some populations there is a seasonal variation in the incidence of type 1 diabetes. For example, in Finland the pattern of autoantibody appearance suggests a potential role for enterovirus infections as triggers of β-cell autoimmunity with noticeable seasonal variation 18.

Etiology and Pathogenesis

In general, autoimmune diseases, including Type 1 diabetes, result from three major interacting components: genetic susceptibility, environmental factors and the immune system 19. Discovery of inflammatory insulitis by Von Meyenburg in diabetic patients and the later finding of an association of this insulitis with the islets of children with T1D

provided the first evidence that this form of diabetes is caused by autoimmunity [2,3]. Although understanding that type 1 diabetes is an autoimmune disorder is an important discovery, the origin of the disease remains poorly understood. One popular model of the natural history of T1D suggests that genetically predisposed individuals with a fixed number of β-cells are exposed to putative environmental triggers that induce β-cell autoimmunity 20. The presence of islet reactive autoantibodies is a marker of ongoing autoimmunity, but activated autoreactive T cells are thought to be responsible for destruction of β-cells. There is always a gap between the onset of autoimmunity and the onset of diabetes, because the clinical symptoms of T1D in general do not appear until approximately 80-90% of the β-cells have been destroyed 21. To understand the pathogenesis and the etiology of type 1 diabetes, it is critical to identify genes that influence the susceptibility to disease, identify environmental agents, and study the role of the immune system and regulatory mechanisms.

Genetics

T1D is complex, multifactorial and inheritance does not follow a Mendelian pattern. There is considerable evidence that genetic factors influence both susceptibility and resistance to type 1 diabetes. Several chromosomal regions have been linked with the disease, suggesting that it is a polygenic disorder. Thus, like most other autoimmune diseases, T1D is influenced strongly by genetic factors. Monozygotic twins have a concordance rate of 30-50%, whereas dizygotic twins have a concordance rate of 6-10%. Children of parents with diabetes have a higher risk of developing T1D compared with the general population, and this risk is higher in children whose father has diabetes (7%) compared with children whose mother has diabetes (2%) 21,22 (Box 1. 3).

Among genetic factors, it is known that genes within the Human leukocyte antigen (HLA) region provide the largest contribution to risk (40-60%) 21. Insulin-dependent diabetes mellitus susceptibility loci 1 (IDDM1) or the Major Histocompatibility Complex, is located within the HLA region on the short arm of chromosome 6 23. There are also non-HLA genes, associated with susceptibility to type 1 diabetes but they are less well understood. Among genetic loci, only some are strongly associated with disease. For example IDDM2, corresponding to the area located upstream of the insulin gene *INS* is believed to contribute 10% of genetic susceptibility. Another well known susceptibility locus is IDDM12, which corresponds to CTLA-4 24. SUMO-4 25, T-bet 26 and toll like receptor 2 27 are some recently identified candidates.

Box 1. 3 Genetic Susceptibility to type 1 diabetes†

General population: 0.4%

Relative: 2-5%

Twin
- Monozygotic: 30-50%
- Dizygotic: 6-10%

Siblings: 5%

Offspring
- Of affected father; 7%
- Of affected mother: 2%

Parents: 3%

†adapted from 21

HLA Class II Region (IDDM1)

The HLA class II genes were shown to be strongly associated with T1D 28-30. These genes code for proteins which are responsible for the presentation of antigens on the surface of antigen-presenting cells (APC) to T lymphocytes 31. The great majority of Caucasian patients have HLA-DR3 or -DR4 class II alleles and approximately 30% to 50% of patients are DR3/DR4 heterozygotes. The DR3/DR4 genotype confers the highest diabetes risk with a synergistic mode of action, followed by DR4 and DR3 homozygotes, respectively [32]. In addition to the HLA complex, several loci within or near this complex can modulate diabetes risk which increases the complexity of the analysis of *IDDM1*-encoded susceptibility 30,33. In spite of the increasing incidence of T1D in the last 50 years, the frequency of patients with high risk HLA genotypes decreased over this period suggesting the role of other factors mainly in the environment 16.

Environmental agents

Genetic susceptibility is a requirement for development of diabetes but in the majority of patients disease expression is thought to depend on interaction with some environmental agents. Viruses, dietary antigens and to a much lesser degree, toxins and stress have the potential to promote diabetes. There is considerable evidence that links diabetes to environment but most of it is indirect, coming from epidemiological and animal studies 29,34. In humans, monozygotic twins share the same risk genes and would be expected to have a concordance of 100 percent for development of diabetes. In practice the concordance is less than 40% overall 18,35 indicating that environment and/or somatic recombination may play a role in development of disease. Concordance of T1D in twins varies depending on age of diagnosis, higher co-incidence (65-70%) when diagnosis occurs

at age less than 5 years and lower co-incidence (18-38%) is observed when diagnosis occurs after 15 years of age. Different environmental factors may explain the difference in development of overt diabetes in twins.

Over the past 50 years, the overall incidence of type 1 diabetes has increased approximately 2-4 fold at a rate of 3% per year in children 0-14 years in the world population 15,16,36. Finland has a six-fold higher incidence of T1D compared with Russian Karelia. These two countries are neighbors and the populations have similar genetic backgrounds and similar frequency of risk HLA genotypes [37]. All of these findings indicate a requirement for genetic susceptibility, which is influenced by environmental factors. Early nutrition and infectious agents, specifically viral infections have been most frequently implicated as early environmental influences [38].

Viruses and bacterial products

Prenatal rubella infection is considered as the only relatively direct evidence, which links diabetes to viral infection. Children infected in the prenatal period developed diabetes in up to 20% of cases [39,40]. In parallel, β-cell autoimmunity was present in 50-80% of cases with congenital rubella syndrome (CRS) [39,41-43]. Although these results indicated that rubella has a role in development of diabetes, current reports indicate the increase of T1D incidence since the 1960s has occurred despite elimination of almost all cases of rubella because of vaccination in developed countries 29.

Enteroviruses that infect stomach and intestine are another suspect for initiation of β-cell autoimmunity [38,44-46]. It has been reported that 40-60 % of newly diagnosed T1D patients have an elevated level of antibodies to Coxackie B viruses (CBV) a subfamily of enteroviruses. In contrast only 0-4% of controls show high levels of antibodies to CBV [47,48].

Because of an amino acid homology between a CBV4 protein and the autoantigen, GAD65, some researchers proposed that CBV can induce diabetes via molecular mimicry [49]. However a review of 26 case-control studies indicated that there was no serological evidence to support a role for the CBV [50]. Islet-related autoantibodies also have been detected after other viral infections such as mumps, measles, chickenpox, and rotavirus [49,51]. Two recent well controlled prospective studies: DAISY in Denver [52] and BABYDIAB [53] in Munich did not show any relationship between CBV and diabetes autoimmunity.

Our knowledge about the role of bacteria, specifically commensal bacteria in development of T1D is limited, and comes from reports of some direct and indirect effects of bacteria and their products on progression of disease. Bacterial products, such as streptozotocin (STZ) and bafilomycin A1, which are produced by Streptomyces species, could be a source of β-cell toxins in humans [54]. Other microbial products such as bacterial LPS, pertussis toxin, cholera toxin, and DNA oligonucleotides with CPG motifs can manipulate the natural immune response to other environmental antigens. These metabolic products have the potential, yet unproven, to change the cytokine secretion pattern and immune response to dietary antigens resulting in a destructive autoimmune response to islets in the pancreas [55].

BB rats and NOD mice, two models of spontaneous T1D, maintained in ultra clean conditions have the highest incidence, suggesting that diabetes incidence is not necessarily dependent on commensal bacteria [56]. Unpublished data using BBdp rats from our laboratory (Lefebvre *et al.*) showed that diet is the major factor affecting development of T1D even in germ-free animals [57]. Animals developed diabetes with a similar frequency either in specific pathogen free (SPF) or strict germ-free (GF) conditions. However, the absence of microbial

factors modified the protective effect of feeding a low antigen hydrolyzed casein-based diet on development of autoimmune diabetes by delaying the age of onset and decreasing incidence compared with animals fed the same diet but maintained under SPF conditions (Lefebvre *et al.*, unpublished data)[57].

Diet and its role in autoimmune diabetes

Food components are considered as non infectious agents that influence the incidence of islet cell autoimmunity and T1D [58]. In the BB rat and NOD mouse models of T1D, diet plays an important role in development of the disease [59-61]. The association between diet and T1D raises the possible role of the gut immune system in the pathology of diabetes. However, our knowledge of the gut and its immune system is based mostly on studies in rodents and therefore remains incomplete. Several dietary factors have been associated with diabetes development 29,34. The two most investigated are discussed below: cow's milk proteins and wheat proteins.

Cow's milk protein

The role of milk in T1D is controversial. Some studies have shown a correlation between early exposure to cow's milk, lack of breast feeding and the development of T1D [58,62-64], but others did not find a correlation [65-67]. A human prospective study, TRIGR (Trial to Reduce IDDM in the Genetically at Risk) [68], investigated the effect of foreign dietary protein in genetically predisposed neonates. Results from the TRIGR pilot study indicated that high-risk neonates weaned onto a hydrolyzed casein based hypoallergenic formula developed autoimmunity less frequently compared with neonates weaned to a standard milk formula [68].

Caseins, beta-lactoglobulin, alpha-lactalbumin, gamma-globulin, and serum albumin are some of the major protein of cow's milk. Caseins are the main proteins in cow's milk

(80%); in contrast, bovine serum albumin represents only 1 % of milk proteins. Studies in Finnish children showed that antibodies to bovine serum albumin were present in all children at onset of diabetes, but were absent in controls [69]. Interestingly, other studies showed that there was no relation between humoral and cellular immunity to alpha- and beta-casein, beta-lactoglubulin and bovine serum albumin [70,71]. Additionally, some clinical data indicate that there was no association between early exposure to cow's milk and β-cell autoimmunity in young siblings and offspring of patients [72,73]. Furthermore, the increased practice of breastfeeding in developed countries is not consistent with increasing incidence of childhood diabetes [74].

Wheat protein

Wheat is a major part of the food consumed in developed countries where T1D incidence is highest. Since the 1960s, wheat consumption has risen almost 5 percent a year in developing countries [75]. Gluten is the main protein component of wheat, consisting of a large macromolecular complex of polypeptides, mostly (80%) gliadin and glutenin proteins. These proteins are not water-soluble and remain after extraction of wheat flour with water, a process that removes most starch, albumin, and globulins. However, complete separation of these proteins based on their solubility is not practical and trace contaminants of albumin and globulin proteins remain [76].

Wheat proteins are harmless in healthy individuals, but in genetically susceptible individuals, they can be immunopathogenic. For example, gliadin proteins can cause immune-mediated damage to the gut in coeliac disease patients and the water/salt-soluble part of wheat proteins can cause IgE mediated Baker's asthma [77,78].

Animal studies have linked wheat gluten with increased risk of autoimmune diabetes [59,79,80]. Studies show that a milk-free, wheat-based diet produced the highest diabetes frequency in BBdp rat and NOD mice [81] confirming several reports of a higher incidence of spontaneous diabetes in animals fed mixed plant based diets in which wheat is the major component [81,82]. In humans, 2-8% of patients with T1D have wheat gluten sensitive enteropathy (Coeliac Disease; CD), a rate that is 5-25 times the frequency of CD in the general population [83,84]. One report indicates a greater reactivity of T cells to gluten among children with newly diagnosed type 1 diabetes compared with control children [85]. A recent study from our laboratory reported immunoreactivity to a homologue of wheat storage globulin protein (Glb1) correlated closely with the destructive immune process that targets the islet β-cells in the pancreas of BB diabetes-prone and diabetic rats [86].

Data from some prospective studies support the role of wheat protein in the pathogenesis of diabetes in humans. For example, the BABYDIAB study showed an increased risk of developing islet autoimmunity associated with early exposure to gluten (<3 months) in offspring of T1D patients. However, this risk did not change for the cases exposed to gluten after 6 months [87]. In another report, data from the DAISY study indicated that children exposed to cereals from age 0-3 months or after 7 months showed a higher risk for developing islet autoantibodies [88]. Interestingly, neither of these studies found a link between early cow's milk exposure and increased islet autoantibodies. Another human trial tested the effect of eliminating gluten from the diet of relatives of T1D patients who had at least two diabetes autoantibodies. However, removing gluten from the diet did not change the frequency or level of autoantibodies in cases, but, there was some improvement in insulin secretion and insulin sensitivity in relatives of diabetic patients [89].

Considering the accumulating evidence that cereal based diets can promote autoimmunity, it is useful to understand the role of the gut and its immune system. This barrier and its immune guardians are the first major site of interaction with environmental antigens including a large concentration and variety of dietary antigens. The gut mucosal immune system has a dual role: it eliminates infectious agents and prevents damage and in addition, it induces tolerance against harmless food and bacteria, a process called oral tolerance. Oral tolerance is a major component of peripheral tolerance and to date the molecular mechanisms are not known [55]. Evidence is building in support of a central role for the gut and gut immune system in development of type 1 diabetes.

Gut and its role in T1D

The link between intestinal immunity and T1D has been suggested by several studies. Reports show that dietary factors can modify diabetes in animal models of autoimmune diabetes and also in humans [59,79,87,89,91]. Islet infiltrating T cells from NOD mice express a gut homing receptor α4β7-integrin and blocking this receptor with specific antibodies prevents diabetes in this model [92,93]. Adoptive transfer of lymphocytes isolated from mesenteric lymph nodes of diabetic NOD mice can cause diabetes in NOD/SCID-mice [94]. In human T1D patients, autoreactive T cells also expressed the gut-associated homing receptor α4β7-integrin [95]. Intestinal biopsy from diabetic patients, who did not have any signs of CD showed a significant increase in HLA class II and ICAM-1 on epithelial cells, indicating intestinal immune activation. Interestingly, following evaluation of a large panel of cytokines in gut biopsies, IL-18 mRNA was significantly higher in T1D patients. In contrast, CD patients showed lower IL-18 mRNA, and IFN-γ and CD25 mRNA expression was higher in CD gut biopsy samples. This study suggested that the gut inflammatory

process in diabetic patients could be different from the inflammatory process in coeliac patients [96].

There are several reports indicating increased gut permeability in human T1D patients [97-100]. Watts *et al.* [101] reported that zonulin, a protein secreted by gut epithelial cells that regulates intercellular tight junctions, plays a key role in the pathogenesis of T1D in BBdp rats. They showed that the endogenous level of zonulin increased 35-fold in the BB rat intestine during the development of insulitis. Blocking of the zonulin receptor reduced the incidence of T1D by 70% and prevented development of islet cell autoimmunity in animals. A recent study from Sapone *et al.*, reported that 42% of the patients had abnormally increased serum zonulin levels and zonulin upregulation was associated with increased intestinal permeability in a subgroup. These data are consistent with the possibility that diet modification with a protective diet (hydrolyzed casein) not only represents a low antigen diet but could prevent increased gut permeability [102,103]. These reports provide a possible link between the gut, exposure to environmental antigens and development of autoimmunity in genetically predisposed individuals.

Immunology

The development of T1D is the result of β cell destruction by an autoimmune process that may take several years. The exact mechanisms of T1D pathogenesis from initiation until the stage of β cell destruction are not clear yet [104]. In general it is believed that several immune cells including macrophages, dendritic cells, B lymphocytes, and T lymphocytes are involved in the β cell specific autoimmune process [3,104-107]. Potential diabetogenic antigens, could be either autoantigens released from β cells and/or environmental antigens processed by antigen presenting cells and presented to T cells in association with MHC class II

molecules. APCs activate Th1 $CD4^+$ T cells mainly by secretion of IL-12 which causes the breakdown of the balance between effectors and regulatory cells[107]. In Th1 conditions, cytokines produced by T cells play an important role in the pathogenesis of T1D [104]. In addition, in Th1 conditions, precytotoxic cells become cytotoxic and cause β cell destruction by different mechanisms. For example, cytotoxic macrophages release IL-1β, TNF-α and IFN-γ and also free radicals [108-110]. $CD8^+$ cytotoxic T cells in association with MHC class I molecules release granzyme and perforin or induce apoptosis using Fas/Fas ligand [111].

The role of T-cells in the pathogenesis of T1D is well accepted [106]. For example, infiltration of islets (insulitis) has been shown to be associated with T cells and administering cyclosporine A, a T cell blocking agent, delayed the disease process. In addition, pancreas transplantation from one non-diabetic twin to the other diabetic twin, provided evidence regarding the role of islet specific memory T cells in destruction of β-cells [112]. T cell infiltration into the pancreatic islets of Langerhans is thought to be a β-cell driven process [106,112,113]. Therefore, immune modulation of the process will be one possible way to prevent the disease either at the initial stages or at the time of transplantation and /or during attempts to stimulate endogenous regeneration (e.g. with GLP-1 analogues). Such preventive strategies will be more feasible as knowledge is gained of β-cell target antigens and/or antigens from the environment that initiate immune dysregulation and cause β-cell destruction.

Humoral immunity

The contribution of antibodies to the pathogenesis of certain autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis is postulated to occur through several different mechanisms such as opsonisation, complement activation, improved

antigen presentation, and other routes [114]. However, to date there is no strong evidence to support a direct contribution of antibodies to β-cell damage [115]. The presence of islet autoantibodies against insulin, GAD65 and IA-2 has been used to predict the risk for development of T1D [115,116]. First-degree relatives of T1D patients, who expressed autoantibodies to three autoantigens have a 75% 5-year risk of diabetes compared with a 25% 5-year risk in relatives who expressed only one autoantibody [117]. Although, islet autoantibodies are useful for prediction, autoantibodies have limited value as surrogate markers for monitoring disease progression and interventions [118].

Cellular immunity

T1D is well known as a T cell-mediated disease, requiring contributions from both $CD4^+$ and $CD8^+$ T cells [106]. Therefore, identifying the antigenic specificity of pathogenic β-cell-reactive T cells is important. Virtually all the candidate autoantigens for disease-associated T-cells were identified originally using autoantibodies in serum of diabetic individuals. β-cell antigens, including GAD65, insulin, and IA-2 were identified using antibodies from T1D serum, and these proteins were found to be targeted by T cells [104,115]. Interestingly, several reports indicate an inverse correlation between T- and B-cell responses to β-cell autoantigens including GAD65, GAD67 and ICA69 in T1D patients [119,120]. In addition, there are other candidate islet autoantigens which could serve as targets for autoreactive T cells that were not discovered through the presence of autoantibodies [113,119,121].

Thus, it is important to develop T cell assays to identify potential autoantigens in T1D. However, there are several difficulties with reliability and reproducibility of cellular assays that limit the use of T cell assays in human or animal models to find surrogate

markers of insulitis. Among the limitations in T cell assays, PBMC are generally the only available source of immune cells in human studies. Therefore, we must expect a very low frequency of specific immune cells in the order of 1 in 100,000 in PBMCs [119]. Traditional techniques for proliferation assays are not sensitive enough to discriminate between background and positive responses from such a limited number of specific T cells. In addition, it is important to use fresh cells as this affects the consistency of results between different studies [119]. On the other hand, purity, concentration of antigen and difference in culture media are other factors that affect the reliability of T cell assays. The development of new techniques such as ELISPOT and HLA tetramer assays are evolutionary steps in the detection of specific T cells. Unfortunately, these sensitive techniques also have limitations related to the toxicity of whole protein, which limits their use in screening. In this study, I used a different approach, a CFSE proliferation assay, which is a sensitive and reproducible technique to discriminate positive and negative T cell responses between controls and patients in response to dietary protein [122].

Coeliac disease and T1D

Coeliac disease or gluten sensitive enteropathy is the most common life long chronic disease, with prevalence in the general population as high as 1 to 1.3% in developed countries [123,124]. The destruction of intestinal villi triggered by an immune response to gluten-containing foods leads to malabsorption and clinical symptoms of the disease.

The pathogenesis of CD is not clear, but development of disease depends on the presence of gluten in the diet and is closely related to specific HLA alleles DQ2 and DQ3 [125,126]. Although 20-25% of the general population carries HLA-DQ2, still only 1% develops CD, indicating a low risk between the presence of HLA-DQ2 and development of disease.

T1D is also considered as a potential gluten-associated disease. The prevalence of CD among children with diabetes is between 1.0 - 6.2% [127-131]. There are two reports of even higher T1D/CD, one from Algeria and the other from Denmark, where prevalence of CD was reported to be 16.4 and 12.3% in children with T1D respectively [132,133]. Of interest, islet related antibodies are present in 23% of patients with CD, and their presence may predict the future development of T1D in these patients [134].

Coincidence of T1D and CD is consistent with the possibility that dysfunction in the gut and its immune system could promote the development of an abnormal immune response to dietary wheat proteins, which may cause coincident development of both diseases. There are reports that in most patients with both diseases, diabetes developed first and CD symptoms developed later [135]. This observation raises the possibility that in cases who developed CD first, a gluten free diet has a protective effect and prevented the development of diabetes [136].

Summary and hypothesis

In summary, the triggers and / or promoters, which stimulate the immune system to attack β-cells and the details of the β-cell destructive process, remain unclear. Previous research both from human and animal studies, indicates that exposure to cereal-based diets can promote autoimmunity or autoimmune destruction of pancreatic β-cells while removing wheat gluten from the diet affects the magnitude of autoimmunity. Removing wheat protein from the diet cannot prevent an ongoing autoimmune process, but could have a benefit in restoring islet mass and function of insulin producing cells [87,89]. Therefore, identifying wheat-sensitive individuals, who are at risk of T1D, is important because it is conceivable

that adopting a gluten free diet could prevent the development of autoimmunity or delay onset of diabetes in these individuals.

Several reports indicate increased gut permeability is present in T1D patients [97-100]. Also, it has been proposed that the zonulin protein that modulates intestinal permeability is increased in a subgroup of type 1 diabetic patients and diabetes prone BB rats [101,137]. In addition, intestinal biopsies from diabetic patients showed immune activation in the gut and the histological pattern of intestinal biopsies was different than CD, suggesting that this activation is not due to coeliac disease [96]. Cells isolated from MLNs of wheat-fed BBdp rats contain an unusually high proportion of Th1 cells that proliferate specifically in response to wheat protein antigens [138]. Therefore, identifying the stimulatory antigens in wheat proteins and finding the origin of potential β-cell specific T cells which initiate β- cell destruction, is an important goal to help clarify the pathogenesis of T1D.

The role of wheat-specific cellular immunity in diabetic individuals is not clear yet and to date little is known about cellular immunity against wheat proteins in peripheral blood of human T1D patients. In humans, Peripheral Blood Mononuclear Cells (PBMC) are the cells that can be obtained by the least invasive procedures to evaluate T cell immune responses. PBMC contain circulating immune cells that represent the pooled immune cells in the whole body. To my knowledge there is only one published report[85] that evaluated the immune response of PBMC to wheat gluten in patients with T1D. In this report, they showed increased T cell response to wheat gluten, but the response was relatively weak, possibly due to the antigen preparation or other technical difficulties with the proliferation assay as noted above [118]. Therefore, further study is required to detect specific T cells and evaluate the immune response of T1D patients to dietary wheat proteins.

In the present studies, I hypothesized that a subpopulation of individuals with T1D has abnormal immune responses to wheat proteins. The major aim of this study was to determine whether there is an abnormal immune response to a mixture of wheat proteins or a purified wheat storage globulin homologue, Glb1 in T1D patients. If so, the question arises regarding the nature of the cytokines produced and whether such reactivity is genetically controlled by specific HLA alleles.

The following are the approaches and sub aims in this thesis:

- To establish a sensitive CFSE proliferation assay to determine the presence of specific T cells that respond to dietary antigens (Chapter 2 and 3).
- To determine whether there is an abnormal T cell immune response to a mixture of wheat proteins (WP) and characterize the nature of the immune response (Chapter 3).
- To determine whether reactivity to WP is associated with specific HLA alleles (Chapter 3).
- To evaluate whether an abnormal T cell response to other dietary antigens is present in T1D patients (Chapter 3)
- To generate and purify a recombinant baculovirus expressing His-Glb1 protein (Chapter 4)
- To evaluate, the immune reactivity to Glb1 homologue in a highly wheat sensitive patient with T1D and coeliac disease (Chapter 5).
- To establish an ELISA assay to measure Glb1 antibody levels in serum and plasma (Chapter 2 and 6)

- To use recombinant His-Glb1 protein to identify Glb1 reactive T cells in a subset of young adults with T1D, to characterize cytokines produced and to determine HLA association (Chapter 6).
- To determine humoral immune responses to His-Glb1 protein in samples from young adults with T1D or children at high-risk (Chapter 6).

Chapter Two

Materials and Methods

Materials and Methods

Patients

Case Study

A 28-year-old Caucasian female with type 1 diabetes and coeliac disease was studied. The patient had the diabetes-associated HLA types DRB1*0401, DRB1*0301, and DQB1*0302, as well as the coeliac associated HLA types DRB1*0301, DQB1*0201, and HLA-DR53. Control subjects (n = 8; 6 female, 2 male) with no significant medical history ranging in age from 24 to 40; mean age 29 ± 6.8 years were investigated. The Ottawa Hospital Research Ethics Board approved the study, and informed consent was obtained before sampling.

Study groups in Ottawa

The study was approved by the Ottawa Hospital Research Ethics Board and Children's Hospital of Eastern Ontario Ethics Board. Patient volunteers were recruited through physicians at the Ottawa Hospital and CHEO, Ottawa, Canada. The subjects have clinically proven T1D. Most were young adults (n=42) of both sexes between 8-41 years of age and including one child (n=1). In addition, 22 unrelated controls without acute infection or autoimmune disease were recruited in a similar age range 18-40 and ethnic group (Caucasian) (Table 1).

Group	Number	Sex (f/m) Ratio	Mean age (Range) (years)	Mean duration (years ± SD)
T1D	42	28/14	27 (8-41)	11.3 ± 7
Control	22	11/11	25 (18-40)	

Table 2.1 Description of Subjects.

Control subjects and patients with type 1 diabetes were recruited. Study groups were age matched.

Case-control study (DAISY, Denver)

We received 151 samples from cases and controls from the DAISY study - The **D**iabetes **A**uto **I**mmunity **S**tudy in the **Y**oung at the Barbara Davis Center for Childhood Diabetes/ University of Colorado Health Sciences Center. Two or more samples were received from 35 cases of strict autoimmunity (one or two islet autoantibodies present). One or two samples were collected before and one sample was collected after they became positive for diabetes autoantibodies. Also from the DAISY study, 63 controls were chosen from children without autoantibodies but at high genetic risk for diabetes. These high-risk controls were frequency matched to the case group on the age of first positive visit and HLA-DR 3/4 status. Age (mean ± SD) was 1.8 ± 2 years for negative visit samples, 3.8 ± 3.3 years for positive visit samples and 4.0 ± 2.4 for controls.

Case-control study (CEDAR, Denver)

We received 217 serum samples from cases and controls to evaluate the Glb1 antibody level from the CEDAR Study - **C**o**E**liac **D**isease **A**utoimmunity **R**esearch in

Denver, Colorado. Samples consisted of two or more serum samples from 50 cases, who were tissue transglutaminase (tTG) IgA positive, which identified them as being high risk for CD. The negative visit sample was obtained before cases became positive for tTG IgA. 50 controls were selected from tTG IgA negative high-risk children participating in the study. The high-risk controls were matched with cases on age, gender, ethnicity, and HLA-DR3. Age (mean ± SD) was 2.2 ± 1.6 years for all negative visit samples, 3.7 ± 2.0 years for the last negative visit sample, and 4.9 ±1.8 years for the first positive visit. For the control group this value was 4.9 ± 1.9 years.

Sample preparation

Isolation of serum or plasma and storage of Ottawa samples

Blood was drawn and transferred into vacutainer serum separation tubes (BD Vacutainer™ SST® Gel and Clot Activator); for PBMC collection whole blood tubes with liquid EDTA (Vacutainer®Blood Collection Tubes, K3 EDTA) were used. Serum was separated from the vacutainer immediately after sampling by centrifugation at 2000 × g for 5 min. We also collected 1:1 plasma diluted with PBS from Ficoll tubes before isolation of PBMC. Samples (plasma and serum) were aliquoted and frozen at -20°C for future analyses.

Isolation of mononuclear cells

PBMC were isolated from blood by density gradient centrifugation over histopaque®-1077 (Sigma-Aldrich Canada Ca No. 1077). Blood was diluted with PBS 1:1; Ficoll (15 ml) was then carefully injected under 30 ml diluted blood in 50 ml sterile BD Falcon™ centrifuge tube (Becton Dickinson) using a spinal access needle (18 x 90 mm, TSK Laboratory Japan). Tubes were spun at 400 × g for 30 minutes at RT with the brake off. The

buffy coat cell layer containing the mononuclear cells was collected using a sterile pipette and transferred to another 50 ml Falcon tube for washing. Cells were washed twice with Hank's buffer (Gibco 14170-112) containing 20 mmol HEPES (Gibco 15630-080) at 250 × g for 10 min at 4-8°C. The total number of PBMCs and viability of cells after staining with trypan blue were evaluated using a hemacytometer.

Freezing, thawing and storage of mononuclear cells

PBMC ($6\text{-}7\times10^6$) /ml were suspended in cold freezing medium containing 90% FBS, 10% DMSO, and transferred to a 1 ml cryotube which was placed in a NALGene™ cryo 1°C freezing container and immediately transferred to a -80°C freezer to freeze slowly at -1°C/min. After 24 h storage at -80°C the tubes were transferred to liquid nitrogen for storage until use. Cryopreserved cells were thawed quickly in a 37°C water bath and then one vial of cells was added to 5 ml of complete medium. The cells were mixed gently with media and cells were pelleted quickly at 400 x g for 10 min. The cells were resuspended in complete medium and diluted to the appropriate concentration for cell culture or phenotyping.

Antigens

Wheat protein

Wheat gluten powder (ICN Biochemicals, Cleveland, Ohio, USA) was digested with α-chymotrypsin (C4129 Sigma, Oakville, ON, Canada) at 100:1 (w/w) in 175 mM Tris-base at pH 7.8 overnight at 37°C according to Arentz-Hansen *et al.* [139]. The partially digested wheat proteins were centrifuged at 1500 × g for 15 min at RT. The supernatant was

diluted 1:1 with PBS and filtered in two steps, first, the suspension was passed through a 1.2 μm Acrodisc Syringe filter and then filtered through a 0.2 μm Acrodisc Syringe filter (Gelman Laboratory) to sterilize the solution. Protein concentration was determined using the Bradford assay [140] and later confirmed with the Bio-Rad protein assay (Bio-Rad, USA). Bovine serum albumin (BSA, Pierce #23209) was used as the protein standard. The wheat protein antigen solution was heat inactivated for 1 h at 100°C and then aliquoted and kept frozen at -20°C until use.

Gliadin

Gliadin powder (Sigma G-3375) was dissolved and stored in 10 mM acetic acid at a concentration of 10 mg/ml (stock solution).

α-gliadin 33-mer

Dr. Hubert Kolb (German Diabetes Research Institute, Dusseldorf) and Dr. Chaitan Khosla (Stanford University, California) provided α-gliadin 33-mer (residues 57-89).

Other antigens and a mitogen: GAD, OVA, Tetanus toxoid and PHA

Recombinant human glutamic acid decarboxylase (GAD) was purchased from Diamyd Diagnostics Stockholm, Sweden. A vial with formulation buffers without GAD was provided to use as a control in T cell assays. Recombinant human insulin (Sigma I-9278), chicken egg ovalbumin (OVA) protein (Sigma A-5503), and Phytohemagglutinin (PHA) (Sigma St. Louis, MO) were from Sigma Diagnostics, St. Louis, MO. Tetanus toxoid (TT) (lot# TAS 307) at a concentration of 2700 LF/ml was purchased from Connaught Medical Research Laboratories of the University of Toronto.

Flow cytometry

Standard flow cytometery protocols were used to evaluate surface markers of fresh PBMC. Briefly, approximately 0.3-0.5×10^6 cells/Falcon tube (35-2053 Becton Dickinson, Franklin Lakes, NJ) were washed once with 0.5-1 ml of FACS buffer (1% BSA (w/v) in Sheath Fluid (Beckman Coulter, Fullerton, CA). After centrifugation at 300 × g for 5 min and removal of the supernatant, the cells were resuspended in 100 µl of FACS buffer containing fluorochrome-conjugated antibodies. Isotype controls and appropriate compensations were used for multi-color staining. After 30 min incubation at 4°C in the dark, tubes were washed once with 0.7 ml of FACS buffer and spun at 300 × g for 5 min. Cells were resuspended in 0.5 ml of FACS buffer and analyzed using the FC500 flow cytometer (Beckman-Coulter, Mississauga, ON, Canada). The data from 2×10^4 cells following appropriate compensation and gating on live cells, as determined by labeling the cells with 7AAD or propidium iodide (PI). Finally, data were analyzed with Cytomics™ RXP analysis version 1.0 software package (Beckman Coulter Inc).

CFSE-based flow cytometric proliferation assay

CFSE labeling

Human PBMC in Hanks buffer containing 20 mM HEPES, without serum (Life Technologies) were labeled with 2 µM 5, 6- CarboxyFluorescein, Succinimidyl Ester (CFSE; Molecular probes). 40 µl of 50 µM (5 mM CFDA stock solution diluted 1:100 in HBSS), was added to each ml of suspension containing 20×10^6 PBMCs. After staining with CFSE, cells were incubated for 20 minutes at 37°C with gentle shaking every 5 min under

low light conditions. Cells were washed twice with cold HBSS medium containing 20 mM HEPES and 5% (v/v) heat inactivated human AB^+ serum (Bioreclamation INC, Hicksville, NewYork).

Cell culture

Labeled PBMC were resuspended (under low light conditions) in RPMI-1640 (Sigma R8758) containing 5% heat inactivated human AB^+ serum (Bioreclamation INC, Hicksville, NewYork), 2 mM L-glutamine (GIBCO 25030-081), 25 mM HEPES (GIBCO 15630-080), 50 μM β-mercaptoethanol and 1% antibiotic/antimycotic (GIBCO 15240-096) to provide a final concentration of 1.2×10^6 cells/well in 24 well plates (Falcon 353047). Various antigens including wheat proteins (chymotrypsin-treated, heat-inactivated wheat gluten, WP (3.2-12.4 μg/well), Glb1 (10 μg/well), gliadin (10 μg/well, Sigma), α-gliadin 33 mer (10 μg/well), insulin (10 μg/well), ovalbumin (Ova, 1 μM), Tetanus toxoid (TT, 2.7 LF/well) or PHA (5 μg/well) were added to bring the volume of each well to 1 ml. Plates were incubated at 37°C in 5% CO_2. On day 3 of culture, 1 ml of 20 IU/ml rhIL-2 was added to each well to a final concentration of 10 U/ml and plates were incubated until the end of day eight at which time some cultures were split as necessary (i.e., PHA). Supernatants were harvested and stored at -20°C for cytokine analyses and cell proliferation was assessed using a CFSE-based, flow cytometric assay.

Flow cytometry analysis and calculation of cell division index (CDI)

Cells were harvested at the end of day 8 and stained with Cy-chrome (ECD) conjugated anti-human CD3 (Immunotech IM2705) as the primary marker for calculation of cell proliferation. In addition to CD3, other labeled antibodies, were used to evaluate

additional surface molecules in selected individuals. Tubes containing unlabelled (control) and labeled (test) cells were analyzed using an FC500 flow cytometer. FACS plots of CFSE-labeled CD3 ECD-stained cultured cells were recorded and raw data were evaluated with Cytomics^{TM} RXP analysis software version 1.0 (Beckman Coulter Inc) to calculate CDI based on a fixed number of 5000 $CD3^{+}CFSE^{bright}$ cells. Cell division index (CDI) was calculated as follows: CDI=number of $CD3^{+}$, CFSE ^{dim} cells with antigen/number of $CD3^{+}$, CFSE ^{dim} cells without antigen (Chapter Three, Figure 3.1)[122].

Inhibition by polymyxin B

Polymyxin B (PmB) sulfate has been shown to bind and neutralize endotoxins such as LPS [141,142]. To test whether our antigens and purified recombinant proteins were contaminated with LPS, PmB (Sigma, Oakville, ON, Canada) was added at a concentration of 10 μg/ml with different concentrations of proteins and LPS. After 30 min incubation, PBMC were added to the culture wells, and proliferation was evaluated as previously described.

Blocking HLA-DR

Anti-HLA-DR clone G46-6, mouse IgG_{2a},κ (5-10 μg/ml) (BD Biosciences Cat No. 555809) and isotype Mouse IgG_{2a},κ clone G155-178 (BD Biosciences Cat No. 554645) were added to the wells containing PBMC (see CFSE proliferation assay procedure for culture conditions). Antigens were added to the wells 30 min later [142]. Cell labeling and cell proliferation assays were followed as described in the proliferation assay section of this chapter.

Cytokine analyses

CBA (Cytometric bead arrays) by BD Biosciences were used to quantify Th1/Th2 cytokines in supernatants from the cells used for the CFSE proliferation assay. In this assay IFN-γ, TNF, IL-4, IL-10, and IL-6 were evaluated. In addition Th1 (IFN-γ) and Th2 (IL-5) cytokines were evaluated using ELISA for our case report (Chapter 6) to verify the levels measured by CBA analyses.

ELISA

Human IFN-γ and IL-5

Cytokine concentrations in the supernatant from the *in vitro* CFSE proliferation assay were determined using a sandwich enzyme-linked immunosorbent assay (ELISA), with two different monoclonal antibodies (mAbs) which recognize different epitopes. Purified rat anti-human IFN-γ (BD PharMingen Cat No. 551221), purified rat anti-human IL-5 (BD PharMingen Cat No.554393), were coated with 50 µl of 1µg/ml primary antibody in coating buffer (Phosphate buffer pH 9.6 $NaHCO_3/Na_2CO_3$) for 1 h at 37°C or overnight at 4°C in 96 well flat-bottom plates (Nunc MaxiSorp Cat No. 439454). The plates were washed 3 times with ELISA washing buffer (PBS containing 0.05% Tween20 Sigma). Plates were blocked with 200 µl of blocking buffer (1% BSA in PBS) for 1h at 37°C or 4°C overnight. After removing the blocking buffer, 100 µl of cytokine standards or samples diluted in dilution buffer (1% BSA in PBS containing 0.05% Tween 20) were added to each well (duplicates). ELISA dilution buffer was used to fill duplicate wells as blank. Plates were incubated overnight at 4°C. The plates were washed 6 times with washing buffer with at least three incubation intervals of 5 min. 100 µl of 0.5 µg/ml biotinylated detection antibody for each

individual cytokine, anti-human IFN-γ (BD PharMingen Cat No. 554550), anti-human IL-5 (BD PharMingen Cat No. 554491), was diluted in dilution buffer and added to each well for 1 h at RT. The plates were washed as in the washing step and incubated with 100 µl/well of 1:10,000 avidin-horse-radish peroxidase (HRP) (BD Pharminogen Cat No. 554058) in ELISA dilution buffer for 45 min at RT. The plates were washed and incubated with 100 µl/well TMB peroxidase substrate (BD Opt EIA Cat No. 555214) to detect cytokine for 10-30 min at RT. The reaction was stopped using 100 µl/well of 1N HCL. Plates were analyzed at 450 nm using a Multiscan Ascent plate reader (Thermo Labsystems, Helsinki, Finland).

Cytometric bead array

Cytometric bead arrays by BD Biosciences were used to quantify Th1/Th2 cytokines in supernatants from the cells used for the CFSE proliferation assay. A human Th1/Th2 CBA Kit (CBA Human Th1/Th2 cytokine kit II, Cat No. 551809) was used according to the manufacturer's instructions to detect IFN-γ, TNF, IL-4, IL-10, and IL-6. Briefly, an FC500 flow cytometer (Beckman-Coulter) was adapted to read tubes from the CBA kit. Cytometer setup beads from the kit were used and an acquisition template was designed specifically for this machine. 50 µl of supernatant were incubated at RT for 2 h with 50 µl mixture of mAb-coated beads and 50 µl of the appropriate phycoerythrin (PE)-conjugated anti cytokine antibody. After incubation, samples were washed once in order to remove excess of detector antibodies. Data acquisition was performed on the FC500 flow cytometer and data from at least 1800 events were captured (300 events for each of the different cytokine beads). To allow quantification of the captured cytokines, a total of 10 concentrations of standard for each cytokine were used ranging from 0 to 10,000 pg/ml. Standard curves were plotted as

cytokine calibrator concentration *vs.* PE-Mean Flurorescence Intensity (PE-MFI) using BD CBA software version 1.4 (BD Biosciences). The concentration of each cytokine in individual samples was determined by comparing the reading from MFI cytokine beads to the standard curve.

Measurement of serum zonulin

A zonulin sandwich enzyme-linked immunosorbent assay (ELISA) was performed [137] in Dr Alessio Fasano's laboratory at the Mucosal Biology Research Center, University of Maryland School of Medicine, Baltimore, Maryland.

Measurement of anti-Glb1 antibody

IgG-Glb1 antibodies were measured using an ELISA technique that I established in our laboratory. The assay was performed as follows: 96 well flat-bottom plates (Nunc MaxiSorp Greiner's Cat No. 439454) were coated with 50 µl of 0.5 µg/well recombinant Glb1 (expressed and purified in our laboratory) in coating buffer (Phosphate buffer pH 9.6 $NaHCO_3/Na_2CO_3$) for 90 min at 37°C. Plates were washed twice with ELISA washing buffer (PBS containing 0.05% Tween20, Sigma). Plates were blocked with 200 µl of blocking buffer (5% human serum albumin in PBS) (Albumin 25% solution, DIN 02223708, Bayer Corporation, Elkhart, IN) for 1h at 37°C or 4°C overnight. After removing blocking buffer, 100 µl of control or test sample were diluted 1:20 in dilution buffer (5% human serum albumin in PBS containing 0.05% Tween 20) and added to each duplicate well. ELISA dilution buffer was used in duplicate wells as a blank. Plates were incubated overnight at 4°C. The next day, plates were washed 6 times with washing buffer with at least three 5-10

min incubation intervals. At least one hour before starting the washing process, the secondary antibody (Anti-human IgG A0170 conjugated with HRP (Sigma, Oakville, ON, Canada) was diluted 1:20,000 in dilution buffer and absorbed with Glb1 1 μg/ml for 1 hour with continuous shaking at RT to block non-specific binding. 100 μl of pre-absorbed secondary antibody was added to each well and incubated for 90 min at RT with slight shaking. The plates were washed and incubated with 100 μl/well TMB peroxidase substrate (BD Opt EIA Cat No. 555214) to detect cytokine for 10-20 min in RT. The reaction was stopped using 100 μl/well of 1M H_2SO_4. The plates were evaluated at 450 nm using a Multiscan Ascent plate reader. To standardize the results, arbitrary units were calculated in relation to the serum value of an individual who displayed high level of Glb1 antibody [143]. The antibody level in this serum was considered as 100 arbitrary units and used as a standard to calculate the value of other samples in the assay. Two other sera with low and intermediate absorbance were used as internal controls in each assay.

Measurement of LPS contamination in protein antigens

The endotoxin content of extracted or purified proteins was analyzed with a chromogen version of the Limulus amebocyte lysate assay (QCL-1000, Endotoxin; BioWhittaker, Walkersville, MD). The manufacturer's instructions were followed and LPS dilutions (Sigma L-4516/ from: 0127:B8) were prepared and used as additional internal controls.

HLA Typing

DNA preparation

Four to six million mononuclear cells from each volunteer (PBMC or PHA stimulated T cells) were harvested and DNA was extracted using a QIAamp DNA mini kit from Qiagen or cells were sent directly for HLA typing to the Ottawa Hospital DNA laboratory. Extracted DNA samples were evaluated for concentration and integrity using UV spectrophotometry (absorption at UV 260 nm and 280 nm) and agarose gel electrophoresis with ethidium bromide staining. DNA was then diluted with 10 mM Tris to obtain a standard concentration and was then frozen at -70°C until used.

HLA Identification

PCR-SSP based typing was performed for some samples using Olerup SSP^{TM} typing kits for HLA-DR and DQ low resolution (Genovision Inc., USA). Briefly, appropriate amounts of DNA and recombinant Taq polymerase (Gibco-Life Technologies, USA) were added to pre-aliquoted primers. PCR was performed according to the manufacturer's instructions. Agarose gel (1.5%) was used to separate amplified PCR products. A 100 base pair marker ladder (Gibco-Life Technologies, USA) was run along with the PCR products during electrophoresis to determine the size of the amplified product. Manual allele assignments were performed based on the kit instructions. The rest of the samples were analyzed at the clinical laboratory of the Ottawa Hospital, Ottawa, ON, for low resolution and high resolution determination in cases which had HLA-DRB1*03, *04 and DQB1*02.

Expression and purification of Glb1

The His-tagged Glb1 gene which was previously constructed in a pET17b vector by Dr. Amanda MacFarlane in our laboratory, was purified after digestion with XhoI/XbaI restriction enzymes. The His-Glb1 nucleotide sequence was cloned into the same restriction enzyme sites of the BacPAK9 transfer vector (BD Bioscience) and the presence of Glb1 was confirmed by restriction mapping. The His-Glb1 gene was transferred to the baculovirus by forced recombination between the transfer vector and BacPAK6 viral DNA. Recombinant virus was isolated, propagated, and amplified in SF21 insect cells. His-Tagged Glb1 was expressed in SF21 insect cells and the expression of Glb1 was confirmed by Western blot analysis. Recombinant protein was purified on a nickel column using an imidazole gradient. Details of the cloning and purification of Glb1 are given in chapter 4 of this thesis.

Statistical analyses

The significance of differences between means among patients and controls was analyzed using the non-parametric Mann-Whitney U-test provided in GraphPad Prism version 4.0 for Windows, (GraphPad Software, San Diego California USA). Significance of differences in frequencies was evaluated using two-tailed Fisher's Exact Test. Pearson's r correlation was used to determine the correlation between WP T cell response and the other dietary or autoantigens using STATISTICA (data analysis software system, version 6.0, 2002 www.statsoft.com). For all statistical analyses a two-tailed p value of < 0.05 was considered significant.

Chapter Three

T cell response to wheat proteins and association with HLA

Introduction

Type 1 diabetes is an immune–mediated disease. The triggers, which stimulate the immune system to attack β-cells and the details of the β–cell destructive process, remain unclear. Nonetheless, evidence continues to accumulate that diet could affect diabetes, further suggesting a possible link between increased intestinal permeability, environmental exposure to non-self antigens, and the development of type I diabetes in genetically predisposed individuals [34,58,68,144,145].

Genetic predisposition is a prerequisite for autoimmune diabetes 30. Among genetic factors, the HLA region provides the largest contribution (40-60%) 21. More than 90% of Caucasian T1D patients carry either the DR3 or DR4 haplotype 20,146. The DQB1*0302 allele present on the DR4 haplotype is strongly associated with T1D and a synergistic effect on T1D risk is observed in DR3/4 heterozygous individuals [147].

There are several reports that link wheat which is known to stimulate various immune responses[77,78,148] with increased risk of autoimmune diabetes [79-81,149]. Wheat is the most abundant source of protein in the human diet and it represents a complex mixture containing at least 1,300 polypeptides [76].

In humans, patients with T1D develop coeliac disease 5-25 times more than the frequency of CD in the general population [83,84]. Two prospective studies in high risk infants showed increased risk of developing islet autoimmunity was associated with early exposure to wheat proteins [87,88]. In addition, a recent study from our laboratory showed immunoreactivity to a homologue of wheat storage globulin protein (Glb1) correlated

closely with the destructive immune process that targets the pancreatic islet β-cells in the pancreas of BB diabetes-prone and diabetic rats [86].

However, apart from epidemiological and animal studies, data are limited on the cellular immune response to wheat proteins in patients with T1D. We hypothesized that a sub-population of individuals with T1D could have abnormal T cell immune responses to wheat protein.

Experimental design and results

In the present study I assessed whether T1D patients demonstrate abnormal T cell immune responses to WP and characterized the nature of this immune reactivity. In parallel, I analyzed the HLA-DR and DQ haplotype in T1D patients and controls to determine whether reactivity to WP was associated with specific HLA alleles.

The most relevant cells to evaluate the cell-mediated immune response are isolated cells from tissues, likely involved in the inflammatory process of T1D, such as, the gut associated lymphoid tissue (GALT), pancreas and pancreatic lymph nodes. Unfortunately these cells can only be accessed by invasive biopsy. These tissues are either easily damaged or do not yield sufficient numbers of cells. As an alternative, PBMCs were used to evaluate T cell immunity. These cells are easily obtained and can be used as a source of immune cells which represent pooled immune cells in the whole body.

We established and optimized a sensitive CFSE proliferation assay in our laboratory to evaluate T cell immune reactivity to a group of antigens including WP (Figure 3.1 and chapter 2). Forty-two T1D and 22 healthy individuals were evaluated for immune reactivity to WP (for description see Table 2.1).

The samples from our study group in Ottawa for T cell proliferation (Chapter 3 and 5) were not run blind. We analyzed at least one control and one case on the same day to minimize variation of results between cases and controls on different days. We used selected case and control specimens to establish conditions for the T cell proliferation assay (e.g. antigen concentration). The data from the optimized assays were included in subsequent analyses.

Results

T cell response to wheat protein in PBMC from T1D patients and controls

To select an appropriate concentration of WP for T cell assays three concentrations of wheat protein (3.1, 6.2, and 12.4 μg/ml) were evaluated. T cell responses to two concentrations (3.1 and 6.2 μg/ml) of WP antigen were significantly higher in the T1D group compared with the controls, whereas response to 12.4 μg/ml of WP was marginally significant (Figure 3.2 A), indicating possible toxicity of WP at this higher concentration. The highest response was to 6.2 μg/ml, therefore, we chose a concentration of 6.2 μg/ml as the optimum concentration. Cells from responders were mainly $CD4^+$ T cells, $CD8^+$ T cells showed very weak responses to different concentrations of WP (Figure 3.2 B). Of the 42 patients with T1D, 20 (47%) had a positive proliferation response to 6.2 μg/μl WP using the control group mean + 3 SD as the cutoff for a positive response (Table 3.1 and 3.2). The CDI in response to WP was different between patients and controls (mean CDI= 32.0 ± 60.0, n=42) versus (mean CDI=4.9 ± 3.2, n=22); (Figure 3.3, p=0.0004; Mann-Whitney U-test). T cell responses to WP did not correlate with duration of disease, but we observed T cell

responses to WP had a moderate negative correlation with age of T1D patients (Pearson's r correlation, r= -0.35, p=0.025) (Figure 3.4 A and B).

T cell response to dietary OVA and selected wheat antigens in a subset of T1D patients

Increased T cell responses were detected in some T1D patients to various antigens including OVA (ovalbumin, a presumably irrelevant dietary antigen), wheat gliadin, α-gliadin 33-mer peptide (a key coeliac related antigen) and the T1D autoantigen, insulin ($p \leq$ 0.03) (Figure 3.3). T cell responses to TT and PHA did not differ significantly between controls and T1D patients (p=0.2 Figure 3.3). There was a positive correlation between WP T cell responses and T cell responses to OVA, gliadin and α-gliadin 33-mer peptide in T1D patients (Figure 3.4 C, D and E: p=0.001, p=0.00001 p=0.0001, Pearson's r correlation). In contrast, correlation between WP T cell response and T cell response to insulin in T1D patients was not significant (p=0.17) (Figure 3.4 F).

IFN-γ and IL-6 are the dominant cytokines in supernatants of WP-stimulated PBMC

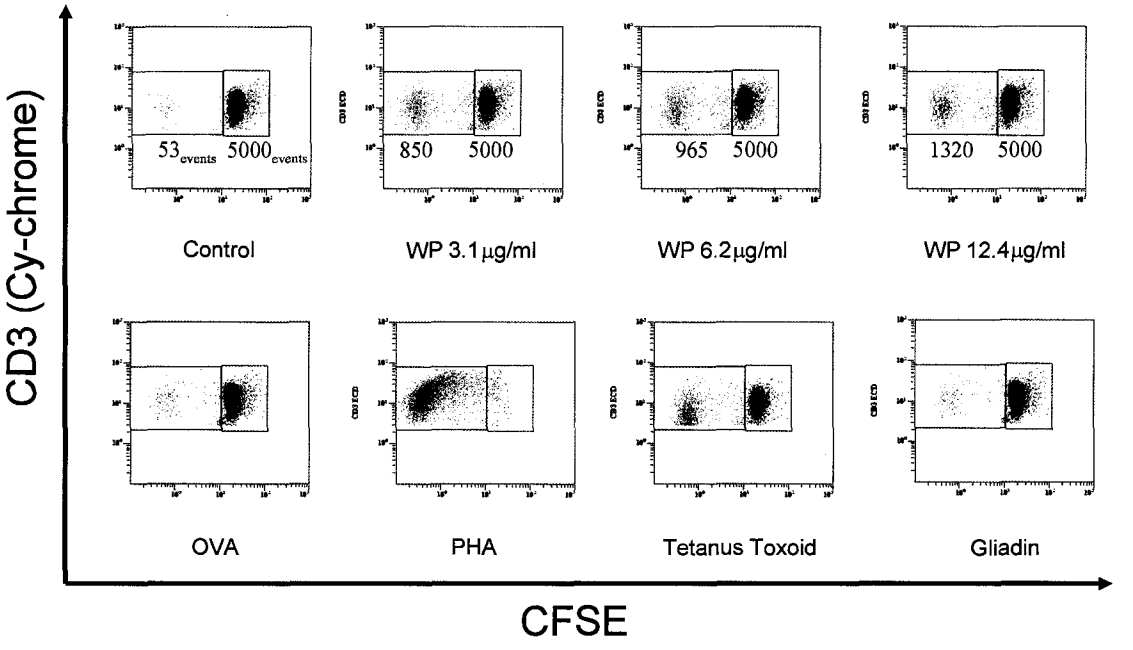
A subgroup of patients with T1D and control subjects was studied for cytokine secretion by WP-stimulated PBMC using flow cytometric bead arrays [150] (Figure 3.5). Results showed a significant difference between control and T1D cytokine levels for IFN-γ, TNF and IL-6 (p=0.005, p=0.007 and p=0.0005 respectively). However after removing one outlier, the mean IL-4 concentration in the culture supernatant was significantly higher in T1D *vs.* controls (p=0.004). It is important to note that the level of IL-4 was much lower compared with IFN-γ (approximately 8 fold lower). IL-10 was produced by WP-stimulated

PBMC at low levels and was not significantly different. Surprisingly, IL-6 secreted by WP-stimulated PBMC was approximately 6 fold higher than IFN-γ.

Figure 3. 1 Antigen-specific $CD3^+$ T cell proliferation

The figure shows FACS plots of CFSE-labeled CD3 Cy-chrome-stained PBMC after 8 d of culture ± antigens. **(A)** 1.2×10^6 CFSE-labeled PBMC from a patient with T1D were cultured for 8 d in the absence or presence of different concentrations of wheat proteins (WP), a chymotrypsin-treated extract of wheat gluten or Gliadin proteins. As a control, cells were cultured with chicken ovalbumin (OVA), tetanus toxoid (TT) or phytohemaglutinin (PHA). On day 8, cells were stained with Cy-chrome conjugated anti-CD3 mAb. The number of $CFSE^{dim}$ events was the number corresponding to 5000 $CD3^+$ $CFSE^{bright}$ events. CDI was calculated using formula in **(B)**.

(A)



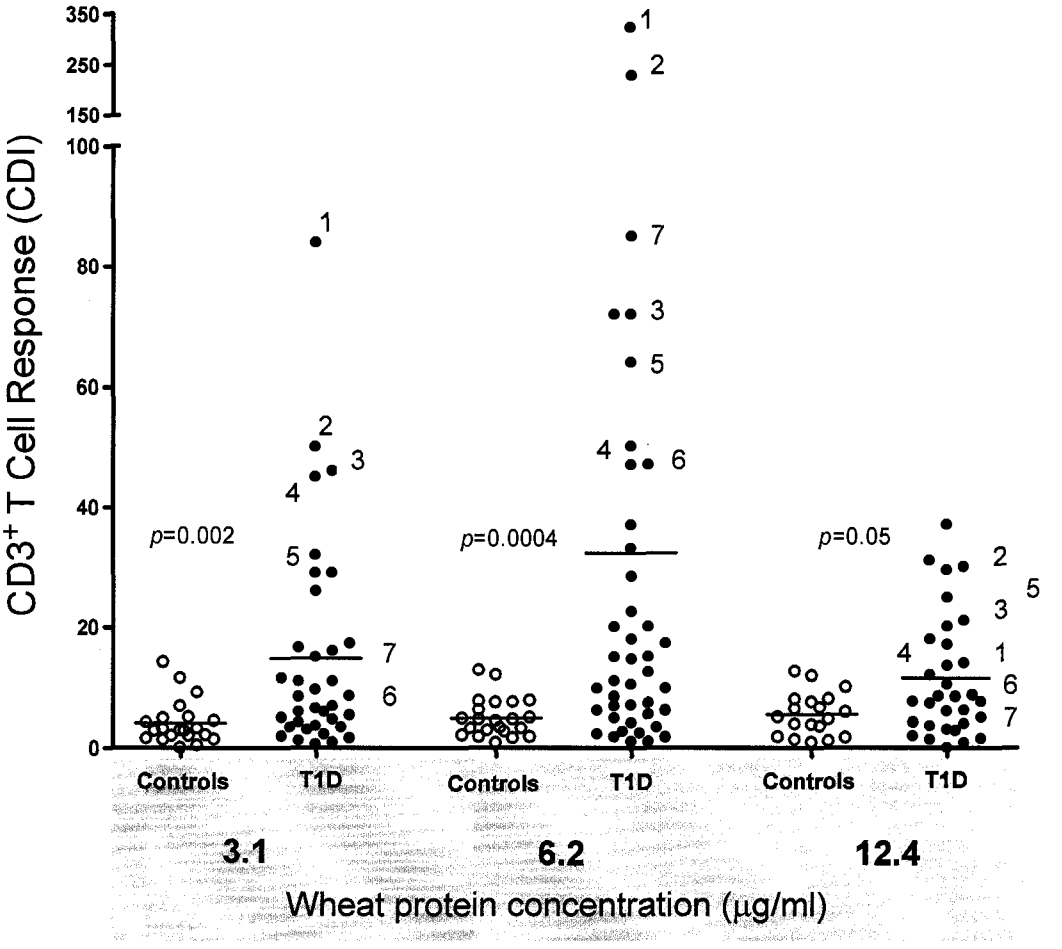
(B)

$$\textbf{CDI :} \frac{\text{Number of CD3}^{+}\text{, CFSE}^{\text{dim}}\text{ cells with antigen}}{\text{Number of CD3}^{+}\text{, CFSE}^{\text{dim}}\text{ cells without antigen}}$$

Figure 3.2 T cell responses to three different concentrations of wheat protein

(A) Response to three different concentrations of α-chymotrypsin-treated heat inactivated wheat protein (WP; μg/ml) was evaluated using a CFSE proliferation assay. Scatter plots show Cell Division Index (CDI) of individual volunteers in response to different concentrations of WP. The means of responses to concentrations of 3.1 and 6.2 μg/ml wheat protein were significantly higher compared with controls (P=0.0004, P=0.002 respectively, Mann-Whitney U-test). The responses to higher concentrations of wheat protein 12.4 μg/ml were marginally significant (P=0.05), suggesting possible toxicity of this dose. Seven individuals who had high CDI in response to a concentration of 6.2 μg/ml are labeled with numbers (1-7) to make it easier to follow their responses at the other two concentrations of WP. **(B)** An example of CD4 and CD8 T cell responses to three different concentrations of wheat proteins in a T1D patient with intermediated immune responses to wheat protein. $CD4^+$ T cells are the major T cells which responded to these WP antigens.

(A)


CD3+ T Cell Response (CDI)
350
250
150
100
80
60
40
20
0
p=0.002
p=0.0004
p=0.05
Controls
T1D
Controls
T1D
Controls
T1D
3.1
6.2
12.4
Wheat protein concentration (μg/ml)

(B)

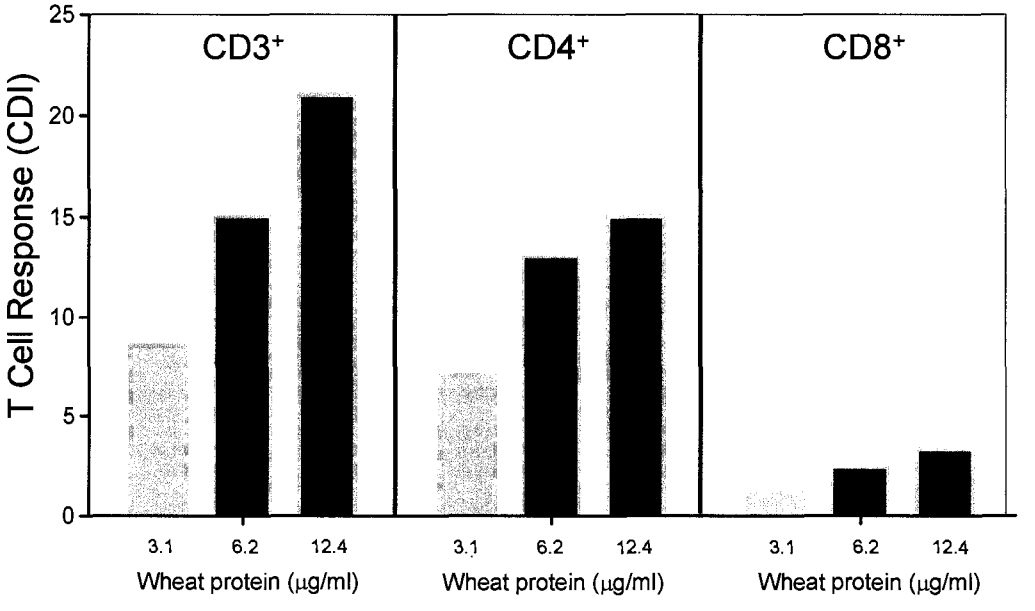

CD3+
CD4+
CD8+
T Cell Response (CDI)
25
20
15
10
5
0
3.1
6.2
12.4
Wheat protein (μg/ml)

Table 3.1 Characteristics of individual subjects in the T1D study group in Ottawa

Status	Subject	Age (y)	Sex	Duration of Disease	T cell reactivity to WP	CDI	T cell reactivity to Glb1	CDI	Antibody reactivity to Glb1	Glb1Ab arbitrary Unit	HLA-DR and -DQ
Type 1 Diabetes	D1	26	F	15	No	6.2	N.D.	N.D.†	N.D.	N.D.	DRB1*04,*13, DR52;DR53; DQB1*06,*03 (DQ8) / DRB1*0401; DQB1*0302
	D2	41	F	6	Yes	20	No	6	No	15	DRB1*04, *07; DR53; DQB1*03 (DQ8,9)/ RB1*0401; DQB1*0302, *0303
	D3	21	F	17	Yes	37	No	5.3	N.D.	N.D.	DRB1*01, *0401; DR53; DQB1*05, *0302 (DQ8)
	D4	29	F		Yes	28.4	No	3.5	N.D.	N.D.	DRB1*03,*04; DR52; DR53; DQB1*02, *03 (DQ8) / DRB1*0401; DQB1*0201, *0302
	D5	8	F	5	Yes	85	N.D.	N.D.	N.D.	N.D.	DRB1*0401, *1303; DR52; DR53; DQB1 *0301 / DQB1*0301
	D6	33	F	8	No	9.8	N.D.	N.D.	N.D.	N.D.	DRB1*04*13; DR52; DR53; DQB1*06,*03 (DQ7) / DRB1*0401; DQB1*0301
	D7	32	F	5	Yes	22.5	No	2	N.D.	N.D.	DRB1*03, *04; DR52; DR53; DQB1*02, *03 / DRB1*0401; DQB1*0201, *0302
	D8	18	F	8	No	6.9	N.D.	N.D.	N.D.	N.D.	DRB1*04, *08; DR53; DQB1*0305, *04 / DRB1*0401; DQB1*0302
	D9	37	F	12	Yes	18	Yes	16	N.D.	N.D.	DRB1*04; DR53; DQB1*03 / DRB1*0401; QB1*0301*0302
	D10	27	F	5	Yes	47	N.D.	N.D.	N.D.	N.D.	DRB1*15,*04;DR53;DR51;DQB1*06,*03(DQ7) / DRB1*0401; DQB1*0301
	D11	22	F	17	No	1.8	N.D.	N.D.	N.D.	N.D.	DRB1*04*13; DR52; DR53; DQB1*06,*03 (DQ7) / DRB1*0401; DQB1*0301
	D12	38	F	7	No	3.4	N.D.	N.D.	N.D.	N.D	DRB1*01/B1*03, DR52, DQB1*02/05 / DQB1*0201
	D13	23	F	7	No	1.8	N.D.	N.D.	N.D.	N.D.	DRB1*03/B1*13, DR52, DQB1*02/B1*06 / DQB1*0201
	D14	19	F	15	Yes	229	N.D.	N.D.	N.D.	N.D.	DRB1*03/B1*04, DR52/53, DQB1*02/B1*03 / DRB1*0401; DQB1*0201, *0301
	D15	20	F	10	Yes	323	N.D.	N.D.	N.D.	N.D.	DRB1*01/B1*04, DR53, DQB1*03/B1*05 / DRB1*0401; DQB1*0301
	D16	25	F	9	No	4.1	N.D.	N.D.	N.D.	N.D.	DRB1*15, *13; DR52; DR51; DQB1*06
	D17	28	F	9	Yes	72	Yes	67	Yes	45	DRB1*0404; DR53; DQB1*0302 (DQ8)
	D18	34	F	30	Yes	17.3	No	1	No	28	DRB1*03,*04; DR52;DR53; DQB1*02,*03 DQ8) / DRB1*0401; DQB1*0201, *0302
	D19	29	F	2	No	10.4	No	1.7	No	24	DRB1*04, *08; DR53; DQB1*03 (DQ7), *04 DRB1*0401; DQB1*0301
	D20	25	F	8	Yes	20	Yes	25	No	38	DRB1*03 (DR17); DR52; DQB10201
	D21	34	F	5	No	8.4	Yes	26	No	32	DRB1*01, *16; DQB1*05; DR51

† N.D. = Not done

Status	Subject	Age (y)	Sex	Duration of Disease	T cell reactivity to WP	CDI	T cell reactivity to Glb1	CDI	Antibody reactivity to Glb1	Glb1 Ab arbitrary Unit	HLA-DR and -DQ
Type 1 Diabetes	D22	19	F	4	Yes	64	No	4	No	28	DRBQ*0404*08; DR53; DQB1*0302 (DQ8),*04
	D23	28	F	15	No	5.5	Yes	22	Yes	49	DRB1*03,*13, DR52, DQB1*06,*02 / DQB1*0201
	D24	36	F	23	No	4.9	Yes	25	N.D.†	N.D.	DRB1*03,*04, DR52;DR53, DQB1*02,*03 / DRB1*0401;DQB1*0201,*0302
	D25	19	F	4	Yes	47	Yes	70	Yes	67	DRB1*03(DR17), 0404; DR52; DR53; DQB1*0201, 0302 (DQ8)
	D26	40	F	27	No	2.7	No	1.1	No	27	DRB1*04, DR53, DQB1*03(DQ7)
	D27	31	F	8	Yes	50	No	5.5	No	26	DRB1*01, *0407; DR53; DQB1*05, *0302 (DQ8)
	D28	19	F	4	Yes	14.7	No	1.0	Yes	48	DRB1*03,*04; DR52; DR53; DQB1*02,*03
	D29	30	M	16	Yes	15	Yes	22	No	20	DRB1*0401*14;DR52; DR53; DQB1*05,*0302 (DQ8)
	D30	21	M	16	No	1	N.D.	N.D.	N.D.	N.D.	DRB1*15,*07; DR53; DR51; DQB1*06,*03 (DQ9) / DQB1*0303
	D31	29	M	19	No	1	N.D.	N.D.	N.D.	N.D.	DRB1*04;DR53;DQB1*03(DQ7)*03(DQ8) / DRB1*0401; DQB1*0301, *0302
	D32	22	M	7	Yes	72	N.D.	N.D.	N.D.	N.D.	DRB1*04/DR53/DQB1*03(DQ8,9) / DRB1*0401
	D33	19	M	10	No	2.43	No	2.1	No	8	DRB1*03(DR17), R0401; DQB1*0201, *0302 (DQ8); DR52, 53
	D34	21	M	4	No	12.5	N.D.	N.D	No	17	
	D35	25	M	9	Yes	33	Yes	30	Yes	51	DRB1*03 (DR17), *0404; DR52; DR53; DQB1*0201, *0302 (DQ8)
	D36	19	M	15	No	11	N.D.	N.D.	Yes	70	DRB1*15; DR51; DQB1*06
	D37	30	M	28	No	9.8	N.D.	N.D.	No	19	DRB1*03, *04; DR52; DR53; DQB1*02, *03 (DQ8) / DRB1*0404; DQB1*0201, *0302
	D38	37	M	7	No	6.2	No	1.1	No	34	DRB1*0401,*09;DR53; DQB1*0301,*0303 (DQ7)
	D39	23	M	11	No	7	N.D.	N.D.	No	12	DRB1*03, *13; DR52; DQB1*06, *02 / DQB1*0201
	D40	28	M	15	No	7.4	No	2.3	N.D.	N.D.	DRB1*0103, *04; DR53/DQB1*05, *03 / DRB1*0401/DQB1*0301
	D41	33	M		Yes	15	No	7.9	No	36	DRB1*03, *04; DR52; DR53; DQB1*02, *03 (DQ7) / DRB1*0401; DQB1*0201, *0301
	D42	25	M		No	2.3	N.D.	N.D.	N.D.	N.D.	DRB1*03, *12; DR52; DQB1*02, *03 (DQ7) / DQB1*0201, *0301

† N.D. = Not done

Table 3.2 Characteristics of individual subjects in the control study group in Ottawa

Status	Subject	Age (y)	Sex	T cell reactivity to WP	CD1	T cell reactivity to Glb1	CDI	Antibody reactivity to Glb1	Glb1Ab arbitrary Unit	HLA-DR and -DQ
Non-diabetic control	C1	23	F	No	1.8	N.D.†	N.D.	N.D.	N.D.	DRB1*15,*07; DR53; DR51; DQB1*06,*03 (DQ9) / DQB1*0303
	C2	26	F	No	0.8	N.D.	N.D.	N.D.	N.D.	DRB1*04, *08; DR53; DQB1*03, *04 / DRB1*0401; DQB1*0302
	C3	26	F	No	12	No	0.8	No	28	DRB1*15*13; DR52; DR51; DQB1*06
	C4	22	F	No	6.2	N.D.	N.D.	N.D.	N.D.	DQ*03(DQ7) DQB1*0301
	C5	24	F	No	1.6	N.D.	N.D.	No	4	DRB1*03,*07; DR52; DR53; DQB1*02 / DQB1*0202
	C6	22	F	No	1.8	N.D.	N.D.	N.D.	N.D	DRB, *13, *15; DR51, DR52, DQB1*06
	C7	18	F	No	12.8	N.D.	N.D.	N.D.	N.D.	DRB1*11;DR52;DQB1*03 (DQ7) / DQB1*0301
	C8	23	F	No	4.7	N.D.	N.D.	No	21	DRB1*16, *14; DR52; DR51; DQB1*05
	C9	18	F	No	7.7	N.D.	N.D.	N.D.	N.D.	DRB1*01,*04; DR53; DQB1*05, *03 (DQ7) / DRB1*0401; DQB1*0301
	C10	22	F	No	3.2	N.D.	N.D.	No	18	DRB1*03, *11; DR52; DQB1*02, *03/ DQB1*0201,*0301
	C11	25	F	No	3.2	No	0.8	No	14	DRB1*15, *04; DR53; DR51; DQB1*06, *03 / DRB1*0404; DQB1*0302
	C12	28	M	No	3.3	N.D.	N.D.	No	21	DRB1*15*13; DR52; DR51; DQB1*06
	C13	26	M	No	7.8	N.D.	N.D.	No	34	DRB1*11;DR52;DQB1*03 (DQ7) / DQB1*0301
	C14	22	M	No	2.9	No	0.2	N.D.	N.D.	DRB1*15, *11; DR52; DR51; DQB1*06, *03 (DQ7) / DQB1*0301
	C15	28	M	No	4.9	N.D.	N.D.	No	16	DRB1*07; DR53; DQB1*02, *03 (DQ9) / DQB1*0202,*0303
	C16	29	M	No	7.5	N.D.	N.D.	No	9	DRB1*15, *04; DR53; DR51; DQB1*06, *03 (DQ7) / DRB1*0401; DQB1*0301
	C17	27	M	No	7.5	No	4.9	No	19	DRB1*01, *0407; DR53; DQB1*05; *0302 (DQ8)
	C18	22	M	No	4.4	N.D.	N.D.	No	20	DRB1*07,*11; DR52;DR53; DQB1*02, *03 (DQ7) / DQB1*0202,*0301
	C19	24	M	No	2.7	N.D.	N.D.	N.D.	N.D.	DRB1*03, *07; DR52, DR53; DQB1*02 / DQB1*0202
	C20	32	M	No	2.1	No	0.2	No	16	DRB1*07, *11; DR52; DR53; DQB1*03 (DQ7,9) / DQB1*0301, *0303
	C21	26	M	No	5	No	3.8	N.D.	N.D.	DRB1*15, *03(17); DR52; DR51; DQB1*06*0201
	C22	25	M	No	4.7	No	4.1	N.D.	N.D.	DRB1*01, *04; DR53; DQB1*05, *03 / DRB1*0401; DQB1*0301

† N.D. = Not done

Figure 3.3 T cell proliferation in response to wheat proteins and other antigens

(A) A CDI value greater than the control mean + 3 SD (CDI >14.6) was used to define a positive response to WP (Red line). 20 patients were designated as responders (48%; p=0.0004 vs. controls). **(B)** The response to OVA in T1D patients was significantly higher compared with controls (p=0.02). The response to the recall antigen, TT and the mitogen PHA as a positive control did not differ between patients and controls. The T cell response to gliadin, α-gliadin 33-mer and insulin was significantly higher in T1D patients (p=0.03, p=0.001, p=0.03 respectively, Mann-Whitney U-test).

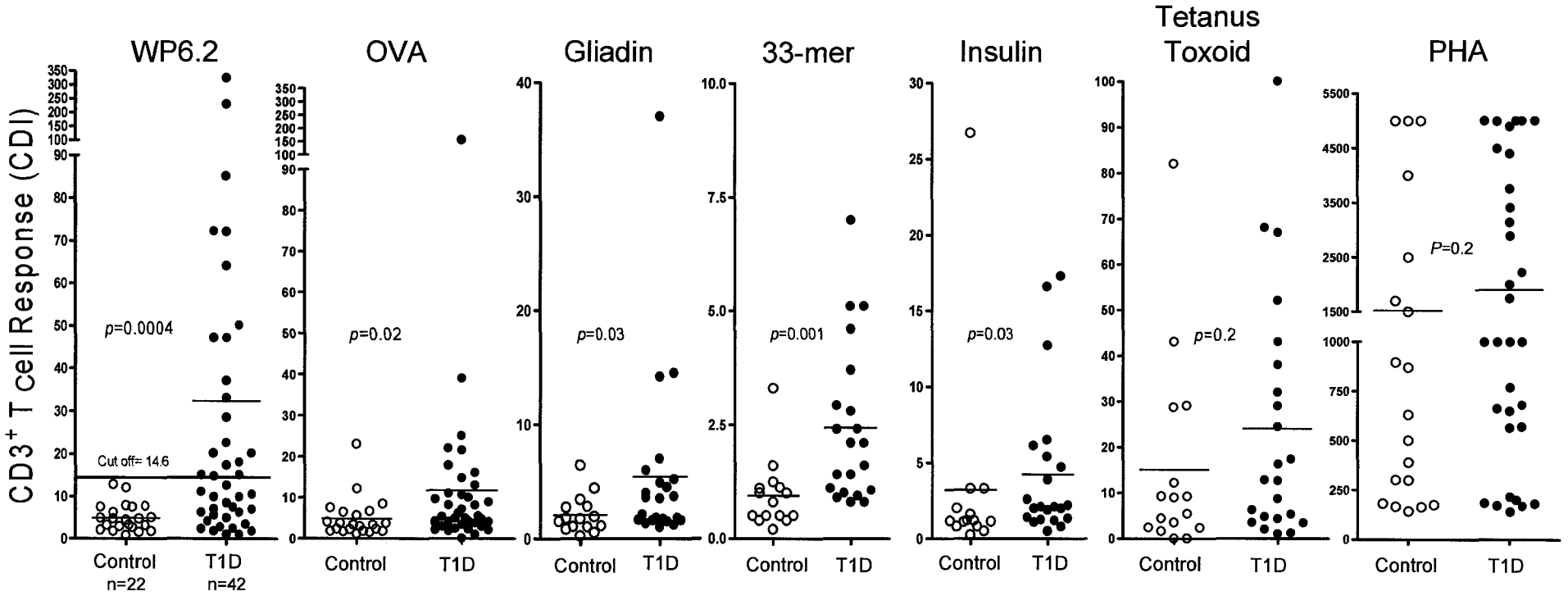
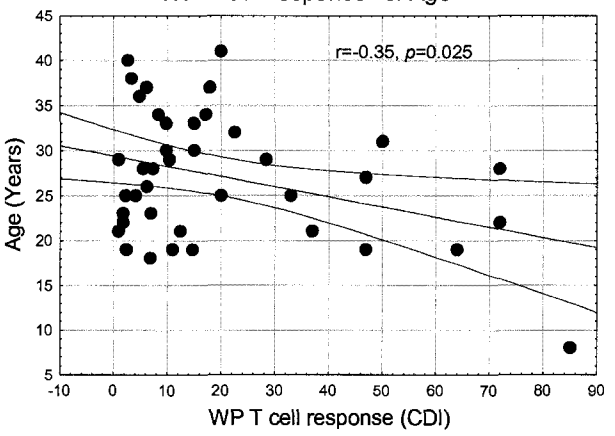

WP6.2
OVA
Gliadin
33-mer
Insulin
Tetanus Toxoid
PHA
CD3+ T cell Response (CDI)
p=0.0004
Cut off= 14.6
p=0.02
p=0.03
p=0.001
p=0.03
p=0.2
P=0.2
Control
n=22
T1D
n=42
Control
T1D

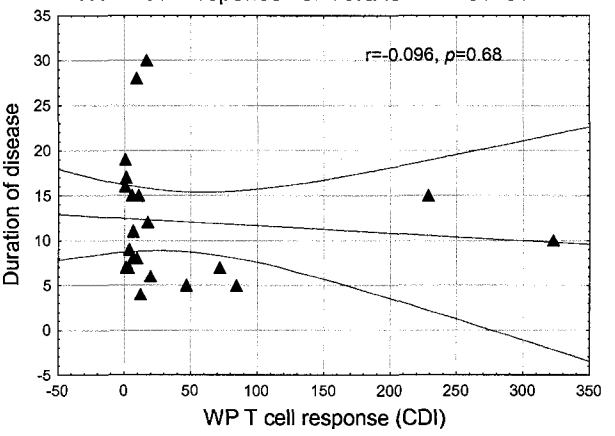
Figure 3.4 Correlation between WP T cell response and age, duration of diabetes and T cell response to other antigens

Results are presented as scatter plots and significance of correlation was determined using Pearson's r correlation analysis. Middle line shows the regression line and upper and lower lines are the regression bands with confidence level of 95%.

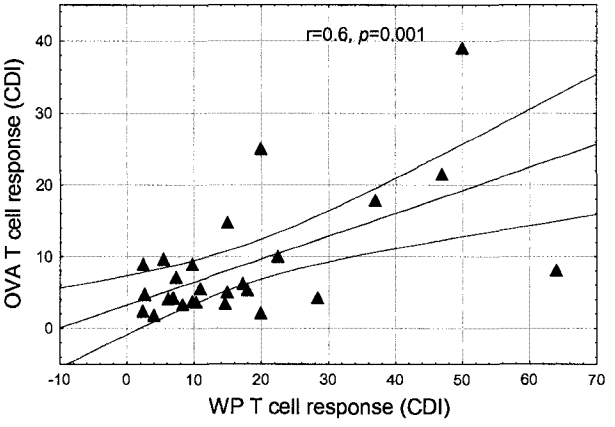
(A)


WP T cell response vs. Age
r=-0.35, p=0.025
Age (Years)
WP T cell response (CDI)

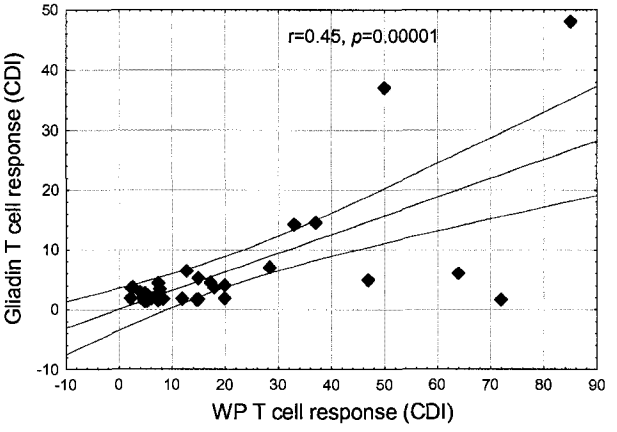
(B)


WP T cell response vs. Duration of disease
r=-0.096, p=0.68
Duration of disease
WP T cell response (CDI)

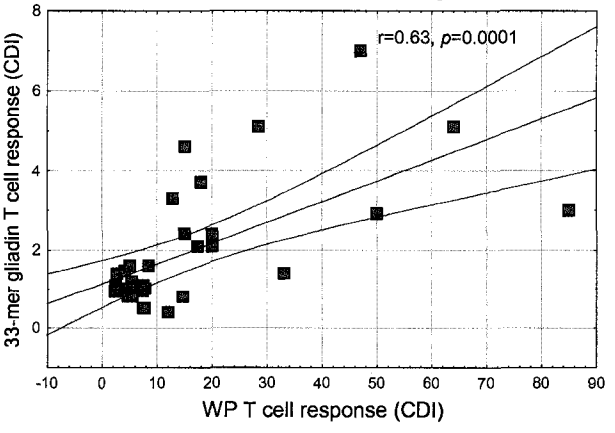
(C)


WP T cell response vs. OVA
r=0.6, p=0.001
OVA T cell response (CDI)
WP T cell response (CDI)

(D)


WP T cell response vs. Gliadin
r=0.45, p=0.00001
Gliadin T cell response (CDI)
WP T cell response (CDI)

(E)


WP T cell response vs. 33-mer gliadin
r=0.63, p=0.0001
33-mer gliadin T cell response (CDI)
WP T cell response (CDI)

(F)

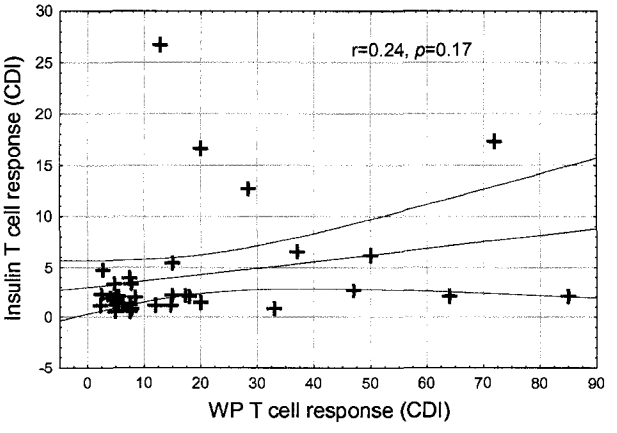
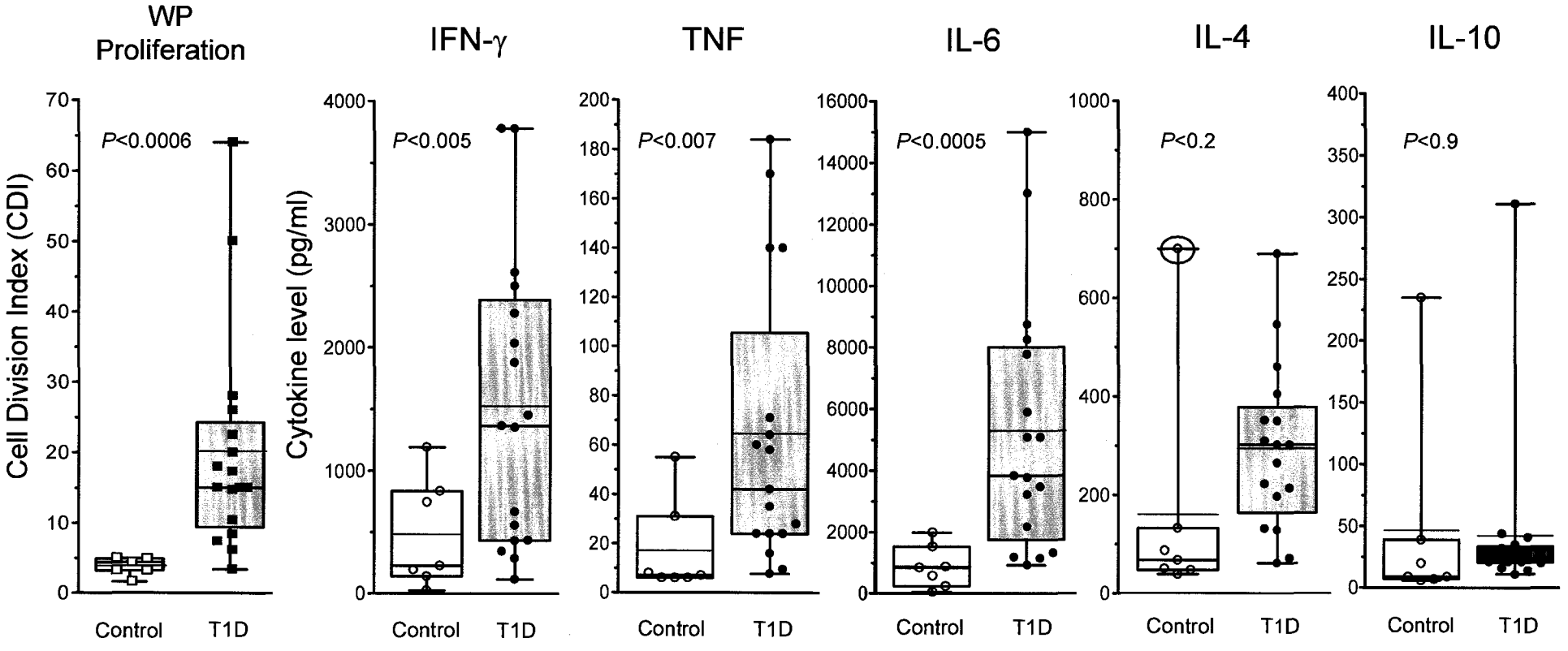

WP T cell response vs. Insulin
r=0.24, p=0.17
Insulin T cell response (CDI)
WP T cell response (CDI)

Figure 3.5 Cytokines produced by WP-stimulated PBMC at day 8 of culture

Box and Whisker plots with individual values for Control and T1D subjects. Each plot shows the mean (red line), median (solid line), distribution, and range. WP-stimulated PBMC of T1D patients showed a Th1 type response, with IFN-γ most abundant. (Note: For IL-4, the difference between control and T1D was significant when the red circled outlier value in the control group was removed $p<0.0004$, Mann-Whitney U-test)


WP Proliferation
IFN-γ
TNF
IL-6
IL-4
IL-10
Cell Division Index (CDI)
Cytokine level (pg/ml)
P<0.0006
P<0.005
P<0.007
P<0.0005
P<0.2
P<0.9
Control
T1D

Immune reactivity against WP is not related to LPS contamination

To understand whether WP was contaminated with LPS like molecules, I measured endotoxin levels with the Limulus amebocyte lysate assay [151] (Table 3.3). The LPS concentration was 0.0012 ng/μg of wheat protein, which is much lower than the acceptable level of endotoxin in T cell assays [152]. To further investigate, I used polymyxin B (PmB), which binds to LPS [153] to rule out the possible effect of LPS contamination in our protein which could affect T cell assays (Figure 3.6). PmB had no effect on T cell responses to WP, whereas LPS induced T cell proliferation was inhibited by PmB.

Association of HLA-DR with immunity to wheat proteins

To determine whether response to WP was associated with the major diabetes risk genes in the MHC class II region, I analyzed HLA-DR and -DQ haplotypes in patients with T1D and controls. The frequency of HLA haplotypes in our control group was similar to published reference values [154]. HLA-DR3, DR4, both of which are associated with T1D, were more frequent in T1D patients compared with controls. Heterozygous DR3/DR4 individuals represented 29% of our T1D patients. In contrast, none of our controls was heterozygous for HLA-DR3/DR4 (Table 3.4 A).

In addition, we compared the frequency of risk alleles in T1D patients who were either responders or non-responders to WP. T1D responders carried HLA-DR4 in 95% of cases, which was significantly higher than the non-responder group, (59%, p=0.01, Table 3.4 B). T cell proliferation in response to WP was analyzed by comparing T1D patients who had HLA-DRB1*03 or HLA-DRB1*04 alone or those who carry both alleles together HLA-DRB1*03/*04 (Figure 3.7A). Only one out of seven T1D patients with HLA-DRB1*03^{+}/*04^{-}

showed a positive response to WP (red circle). In contrast, T1D patients who carried one HLA risk allele DRB1*04 (10 of 20) or patients who carried both HLA risk genes, DRB1*03/*04 (9 of 12) showed reactivity to WP. The frequency of the HLA-DQB1*02, the major Coeliac disease associated allele, was not significantly different between T1D patients and controls (p=0.6) (Table 3.4 A). Importantly, we did not detect any significant differences for HLA-DQB1*02 between responders and non-responders (p=0.75: Table 3.4 B).

To understand whether WP T cell responses were associated with specific HLA alleles, we also evaluated the HLA-DR and -DQ haplotypes by high-resolution analysis in T1D patients. Almost all patients carried *0401 or *0404 and HLA-DQB1*0301 or *0302. However, HLA DRB1*04 was present in 95% of WP responder patients and this frequency was higher compared with non-responders. Unfortunately, HLA high resolution analysis did not permit us to differentiate responders from non-responder (Figure 3.7 A and B).

T cell responses to WP inhibited by monoclonal anti-DR IgG antibodies

To further confirm the importance of HLA-DR in wheat-induced T cell responses, I evaluated the blocking effect of anti HLA-DR mAb on PBMC of 14 T1D patients. Anti-DR antibodies blocked T cell responses to wheat proteins in all T1D patients. Addition of the isotype control antibody did not prevent the T cell response to WP (Figure 3.8). In contrast, HLA-DR mAb only partially blocked T cell responses to the recall antigen TT (Data not shown). Analysis of cytokines in the culture supernatants from WP-stimulated PBMC of T1D patients demonstrated that anti-DR antibody significantly inhibited secretion of Th1 cytokines but not Th2 cytokines (Figure 3.9). Interestingly, IL-4, IL-10 and IL-6 cytokine levels in culture supernatants of WP stimulated PBMC did not show significant differences

when responses were blocked with anti HLA-DR antibody. This raised the possibility that somewhat increased IL-4 in T1D patients (Figure 3.5) came from bystander cell secretion of Th2 cytokines.

Table 3.3 LPS concentration in T cell antigens

LPS concentration of extracted and purified proteins was analyzed with a chromogen version of the Limulus Amebocyte Lysate assay (BioWhittaker kit,Walkersville, MD). The concentration of LPS was calculated and reported as ng/μg of protein. Protein concentration measured by Bio-Rad protein assay (Bio-Rad, USA).

Sample	Source of protein	Protein concentration (μg/ml)	LPS concentration (ng/μg of protein)
Wheat protein (WP)	Our lab Chymotrypsinized WP	4000	0.0012
Glb1	Our lab Purified from insect cells	390	0.0029
Prison protein	Commercial Purified from *E. coli*	100	0.107
rh Insulin	Sigma	10,000	0.000021

Figure 3.6 Polymyxin B did not inhibit wheat protein induced T cell responses in T1D patients

Response to WP (6.3 μg/ml), and LPS (1 μg/ml) and PmB (10 μg/ml) was evaluated with CFSE proliferation assay in a subset of T1D patients. PmB was added at a concentration of 10 μg/ml to each well, 30 min before adding PBMC. PmB did not induce proliferation in control wells. PmB did not prevent T cell proliferation in response to WP. In contrast, PmB inhibited LPS induced T cell proliferation when added to LPS wells 30 min before adding PBMC. Note: linked symbols are from the same patient.

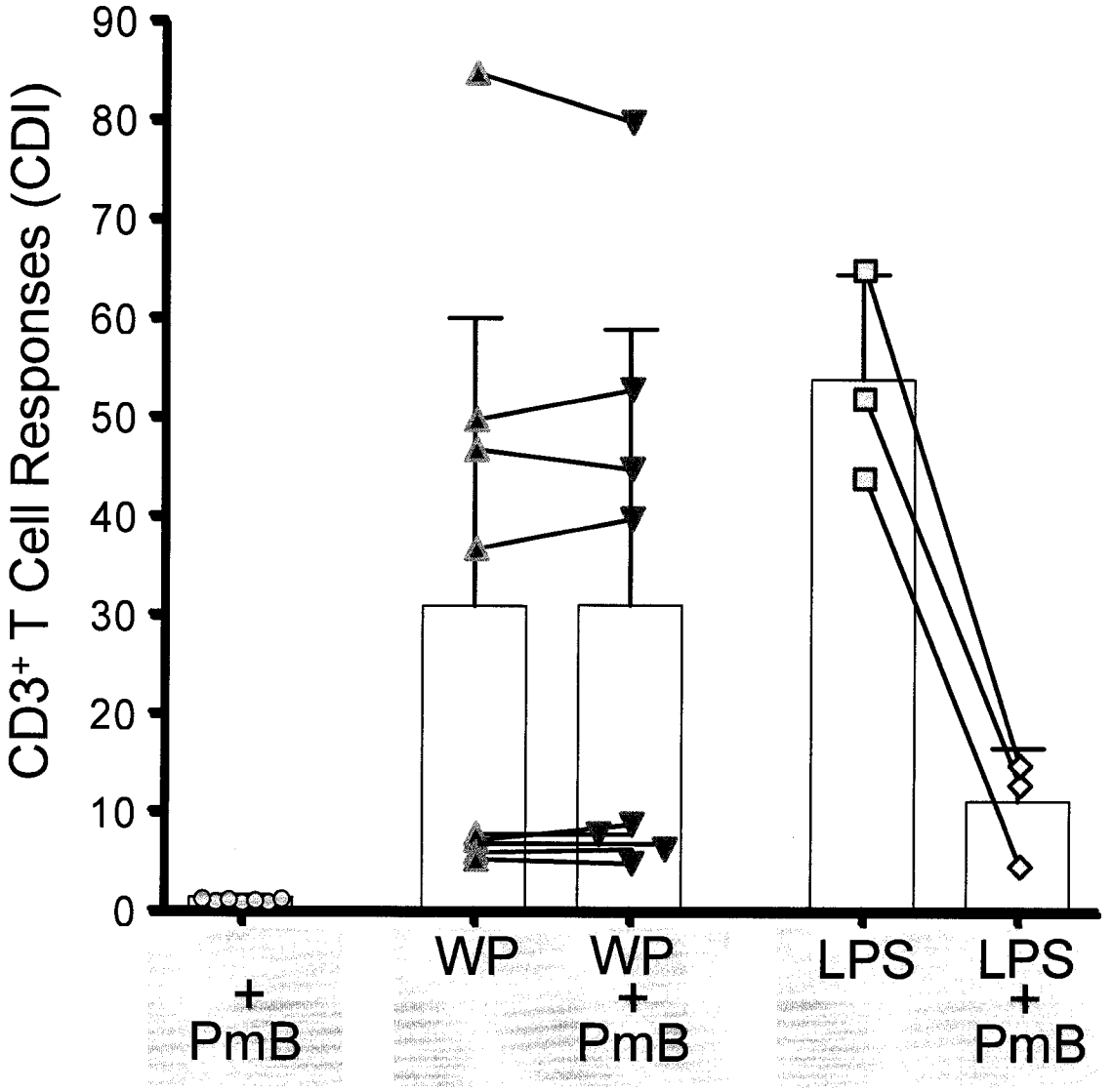

CD3+ T Cell Responses (CDI)
90
80
70
60
50
40
30
20
10
0
+
PmB
WP
WP
+
PmB
LPS
LPS
+
PmB

Table 3.4 Frequency of MHC class II risk genes, HLA-DR and DQ in control subjects and T1D responders, non-responders

HLA-DR and DQ haplotypes of subjects were characterized by PCR-based HLA class II tissue typing. **(A)** The frequency of HLA-DR3 and DR4 was higher in T1D patients. 30% of T1D patients were heterozygous for DR3/DR4; in contrast, none of the controls was DR3/DR4. **(B)** The frequency of HLA-DQ2 genes in T1D responders was not different compared with T1D non-responders. This demonstrated that reactivity to WP was not necessarily related to the coeliac disease associated gene, HLA-DQ2. HLA-DR4 was more frequent among T1D responders compared with non-responders.

(A)

	Subjects (n)	DR3	DR4	DR3/DR4	DQ2	DQ3
Healthy Control	22	23%	27%	0	36%	63%
T1D	42	45% (p=0.1)	76% (p=0.0002)	29% (p=0.005)	46% (p=0.6)	73% (p=0.38)

(B)

	Subjects (n)	DR3	DR4	DR3/DR4	DQ2	DQ3
T1D Non Responder	22	41%	59%	14%	41%	63%
T1D Responder	20	50% (p=0.76)	95% (p=0.01)	45% (p=0.04)	50% (p=0.75)	90% (p=0.07)

Figure 3.7 Relation between wheat-induced T cell response and HLA diabetes risk alleles

(A) Distribution of T cell proliferation (CDI) to WP in T1D patients with HLA-DRB1*03, HLA-DRB1*04 or those heterozygous for HLA-DRB1*03/*04. A CDI value greater than mean + 3 SD of the control group (CDI>14.6) was used to define a positive proliferation response. One of the T1D patients with HLA-DRB1*03^{+}/*04^{-} showed positive responses to WP (red circle). In contrast T1D patients who carried one risk allele HLA-DRB1*04 (10 of 20) or patients who carried both risk genes, HLA-DRB1*03/*04 (9 of 12) showed positive reactivity to WP. High resolution results of HLA-DR are labeled beside each individual. **(B)** Distribution of T cell proliferation (CDI) to WP in T1D patients with HLA-DQB1*02, HLA-DQB1*03 or those heterozygous for HLA-DQB1*02/*03. High resolution result of HLA-DQB1*02 and *03 is labeled beside each individual (↓ 0201 means all individuals in the column carry -DQB1*0201).

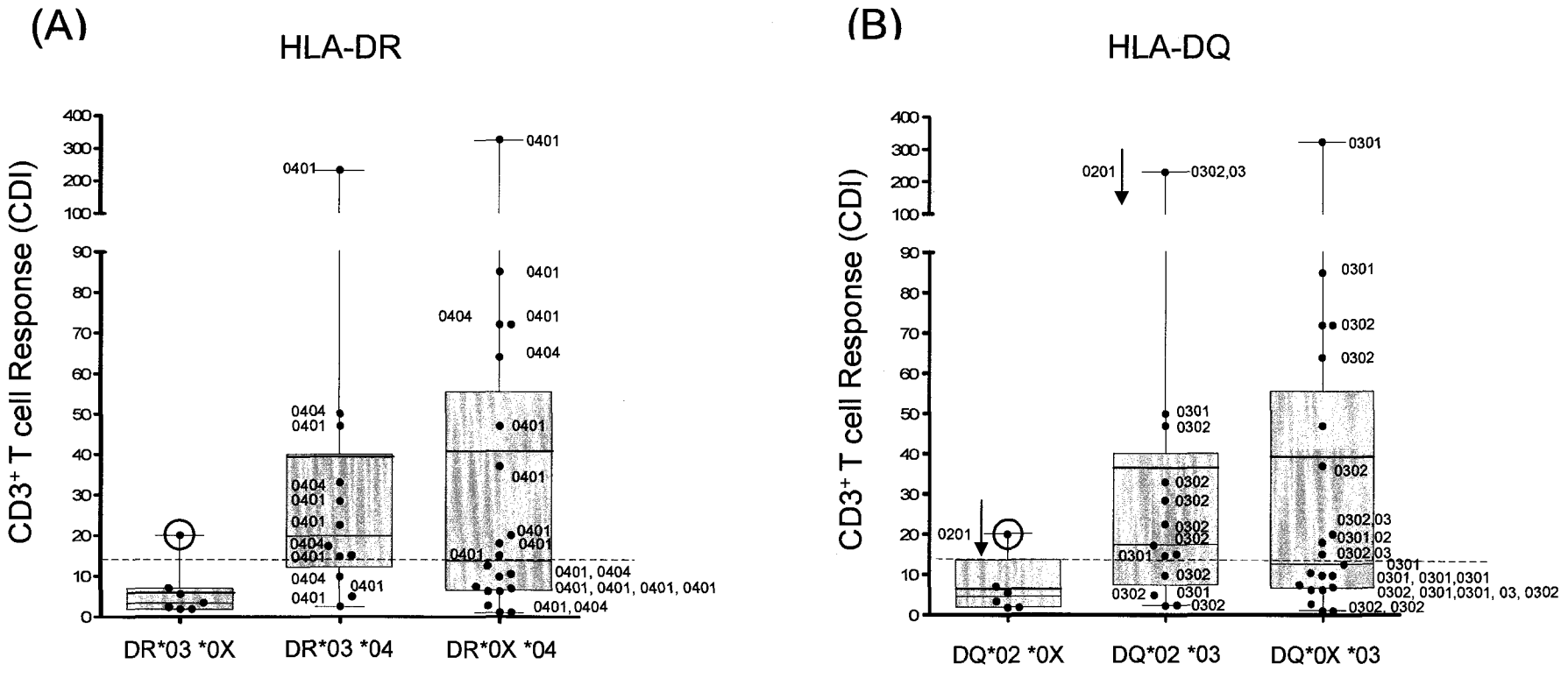
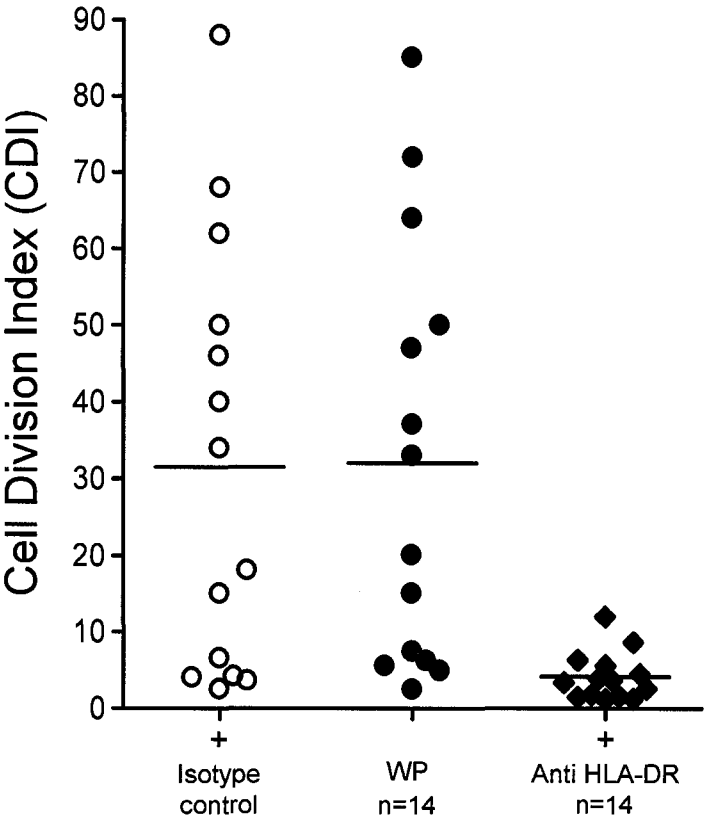

(A)
HLA-DR
CD3+ T cell Response (CDI)
DR*03 *0X
DR*03 *04
DR*0X *04
(B)
HLA-DQ
CD3+ T cell Response (CDI)
DQ*02 *0X
DQ*02 *03
DQ*0X *03

Figure 3.8 HLA-DR antibodies block T1D T cell response to wheat proteins

(A) Anti HLA-DR mAb (5μg/ml) blocked wheat-induced T cell response to wheat protein (6.2 μg/ml) in 14 T1D patients. The isotype control antibody did not affect WP-induced T cell response. **(B)** An example to show complete inhibitory effect of anti HLA-DR antibody in response to wheat protein antigens. T cell responses to tetanus toxoid were only partially inhibited by Anti-HLA-DR antibody.

(A)


Cell Division Index (CDI)
90
80
70
60
50
40
30
20
10
0
+
Isotype control
WP n=14
+
Anti HLA-DR n=14

(B)

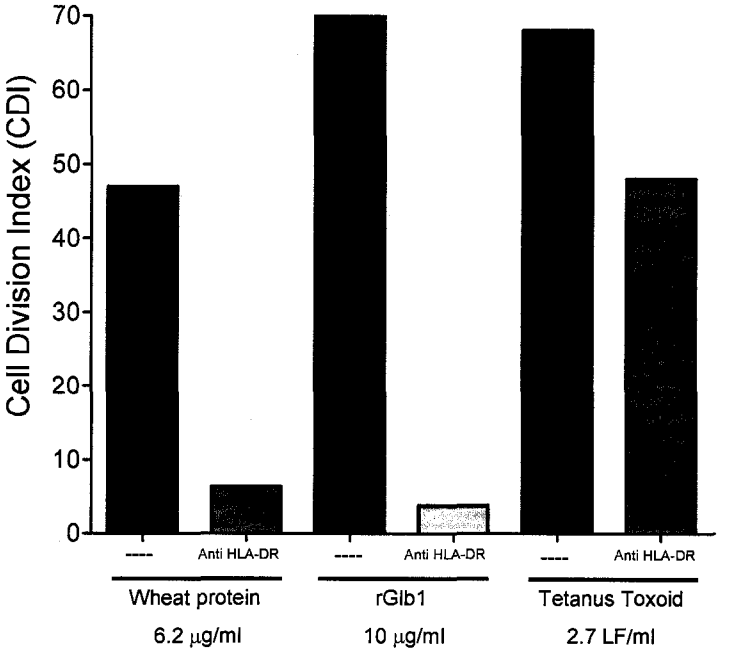
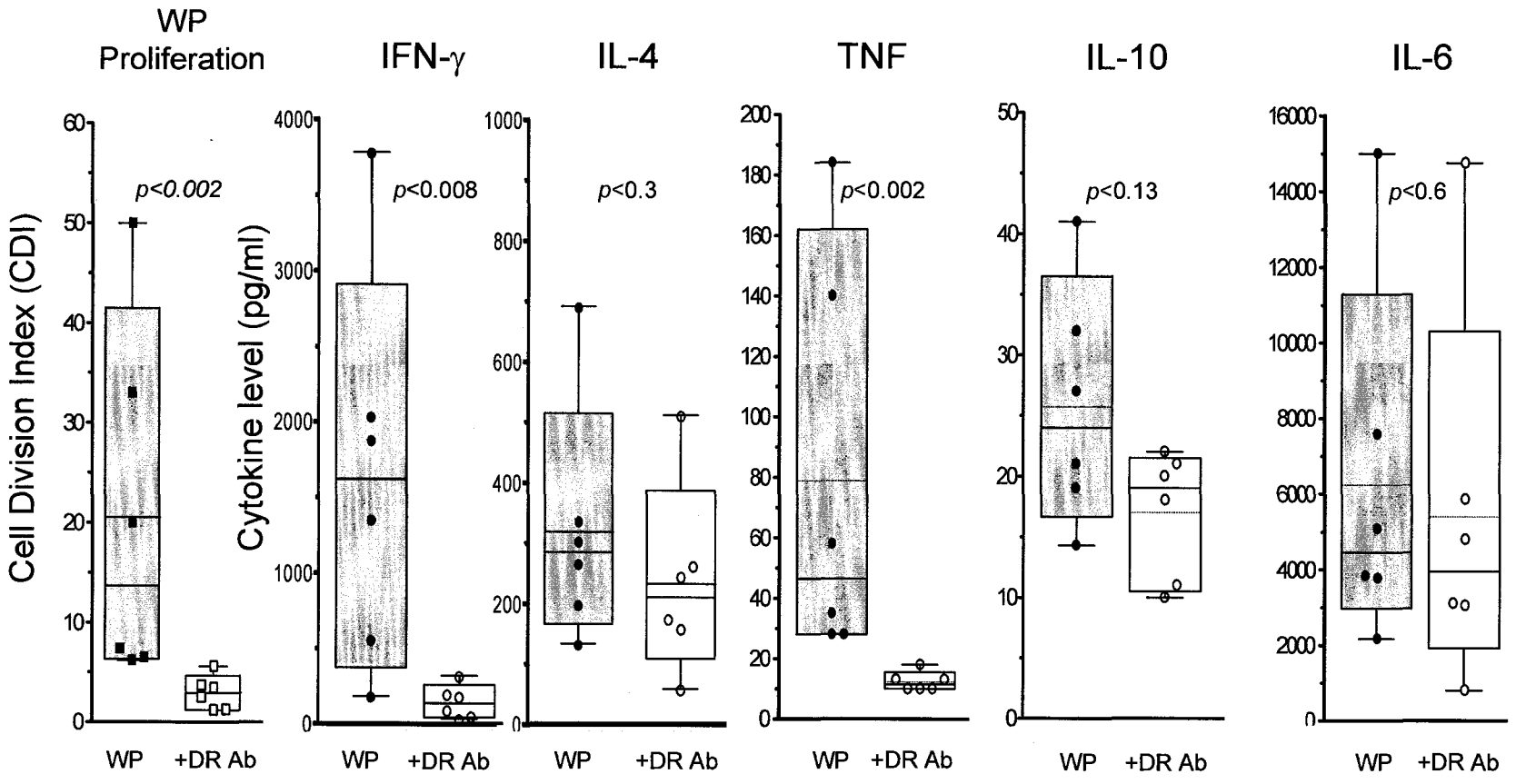

Cell Division Index (CDI)
70
60
50
40
30
20
10
0
---- Anti HLA-DR
Wheat protein
6.2 µg/ml
---- Anti HLA-DR
rGlb1
10 µg/ml
---- Anti HLA-DR
Tetanus Toxoid
2.7 LF/ml

Figure 3.9 Anti HLA-DR antibody blocks secretion of wheat-induced Th1 cytokines

Cytokine profiles of supernatants from WP-stimulated PBMC of T1D patients were evaluated in the absence and presence of monoclonal HLA-DR antibodies using Th1/Th2 cytometric bead arrays. This analysis demonstrated significant inhibition of Th1 cytokine secretion but no inhibition of Th2 cytokines. Note: Blocking with HLA-DR did not affect IL-6 cytokine secretion.


WP Proliferation
IFN-γ
IL-4
TNF
IL-10
IL-6
Cell Division Index (CDI)
Cytokine level (pg/ml)
p<0.002
p<0.008
p<0.3
p<0.002
p<0.13
p<0.6
WP
+DR Ab

Discussion

In this study, T cell responses to wheat proteins (chymotrypsin-treated, heat-inactivated wheat gluten) were examined using a sensitive, reproducible CFSE proliferation assay. Some previous reports suggested that specific diets could promote or protect susceptible individuals from development of diabetes [34]. Wheat is a major amino acid source in the diet of diabetes-prone BB rats and NOD mice and these animals develop diabetes 2-3 times more frequently in comparison with animals that received a protective hydrolyzed casein-based diet [79,91]. In addition, other studies showed that gluten-free diets are protective in NOD-mice compared with gluten-containing diets [81,82]. In studies of high risk infants, early exposure to cereals, particularly wheat has been linked to the appearance of islet autoantibodies [87,88]. The present study demonstrates high T cell response to WP in more than 45% of T1D patients suggesting that immune response to dietary WP is abnormal in a large subset of patients and possibly reflects a role for WP or gut defects in the pathogenesis of T1D.

A previous report showed enhanced cell-mediated immune response against wheat gluten in newly diagnosed T1D patients, however responses in patients and controls in this study were low [85]. I am not aware of other reports that show specific T cell responses to gluten in T1D patients. There are some reports indicating both high and low T cell responses to gluten in healthy control subjects [155-157]. This variation likely reflects the natural variation in response to dietary antigens and variability in T cell assays. For example, variability in antigen preparation, concentration, and T cell assay protocol could affect outcomes [119].

To achieve a sensitive and reproducible protocol to evaluate T cell proliferation in response to WP antigens, we standardized our approach by focusing on three important

aspects: antigen preparation, antigen concentration, and establishing a sensitive proliferation assay.

Wheat gluten proteins are mostly insoluble and sticky and require enzymatic hydrolysis for solubilization [158]. In our study, we used chymotrypsin-treated heat-inactivated wheat proteins. It has been reported that chymotrypsin proteolysis of gliadin at neutral pH can increase wheat protein solubility in water [158]. Klemetti *et al.* [85] used fraction III of pepsin and trypsin (PT) hydrolysate of wheat gluten. PT digestion requires low pH which causes digested protein to become insoluble. In addition, PT digestion would produce different peptides compared with the chymotrypsin procedure we used [158].

Gliadin by itself has a direct cytotoxic effect on cells cultured *in vitro* [159]. It is important to note that our wheat protein extract was clearly cytotoxic at concentrations higher than 25 μg/ml (data not shown). We used the optimal concentration of 6.2 μg/ml of wheat protein after analysis of the dose response relationship (Figure 3.2). In contrast, previous reports used much higher concentrations of gluten: 400 μg/ml in the paper by Klemetti *et al.* [85] and 600 μg/ml was used by Gjertsen *et al.* [160]. It is possible that this amount of antigen was toxic or at least inhibitory to the cultured cells [159]. In addition, the gluten fraction that they used did not contain all gluten proteins. They used fraction III of gliadin, which contains only 75% of gliadin after pepsin and trypsin enzymatic digestion. Therefore, it is likely that the toxicity and/or antigenicity of gluten was eliminated or decreased during the isolation procedure.

Most previous studies used standard thymidine incorporation assays. Unlike flow-based assays, the thymidine incorporation assay does not provide any information on the phenotype of the proliferating cells. Furthermore, this assay measures DNA synthesis in a

short period of time usually the last 8-18 h of the 4-7 day culture period. Therefore, cells that have stopped proliferating and have entered apoptosis before the last day of incubation with thymidine are not detected. The peak responses can be different in response to different antigens and can even differ among individuals [122,161]. Hence, in the present study, we evaluated proliferation of T cells using the CFSE proliferation assay. This technique is more sensitive than the thymidine incorporation assay and it can detect rare antigen specific T cells at a 10 fold lower concentration of antigen 38,122,162. The CFSE dye used in this assay permitted us to monitor 9-10 cell divisions by flow cytometry. In addition, by using this technique we were able to track proliferation of antigen-specific T cells in response to wheat protein using anti-CD3 antibodies. We adapted this technique for a culture period of 8 days with the opportunity to detect the proliferation of specific $CD3^+$ T cells as described in chapter 2.

The inflammatory process in the pancreas which causes the destruction of beta cells, is mainly a Th1 response characterized by the production of IFN-γ in diabetes-prone rodents and humans [79,163-166]. The origin of this response and the stimulatory antigens involved are not well understood. An earlier report from our laboratory indicated a diet–related Th1 bias in the GALT of BBdp rats, associated with increased T-bet/Gata-3 expression ratio and a high frequency of IFN-γ^+ T cells in the MLN of animals fed a standard cereal-based diet in which wheat protein was the main component. In this thesis, cytokine analyses of culture supernatants from cells of human T1D patients stimulated with WP showed a Th1 type response with high concentrations of IFN-γ and significantly higher TNF compared with controls. We also detected high concentrations of IL-6 in supernatants of WP stimulated cultures which were 5-6 fold higher compared with the concentration of IL-6 in controls

(Figure 3.5). Interestingly, anti-DR antibody did not inhibit secretion of IL-6 (Figure 3.9). This observation raises the possibility that IL-6 was secreted from other cell types present in PBMC and suggested that IL-6 production is not HLA-DR restricted. The presence of an abnormal Th1 specific T cell response to dietary wheat proteins in T1D patients raises the possibility that Th1 cytokine-producing cells are activated in association with a coeliac-like pro-inflammatory condition in the gut [167] and could play a role in the dietary modification of T1D.

Several reports indicate a role for IL-6 in diabetes development in NOD mice and BB rats [168-171]. For example, anti-IL-6 antibody inhibits the development of T1D in NOD/WEHI mice [171]. Further, over expression of IL-6 in pancreas caused islet inflammation and induced B-cell differentiation, which may be important in the development of autoimmune disease [172]. We observed very high concentration of IL-6 from PBMC in response to WP (4-6 fold higher than IFN-γ concentration). This observation is in keeping with previous reports proposing a possible mechanism by which wheat can promote the development of diabetes involving induction of pro-inflammatory cytokines such as IL-6, IFN-γ and TNF-α by wheat antigens [34,138].

The human leukocyte antigen genes are the major genetic determinant of T1D. More than 90% of Caucasian T1D patients carry either the HLA-DRB1*03 or -DRB1*04 haplotype [154] and a synergistic effect on T1D risk is observed in DR3/4 heterozygous individuals [147]. Our data demonstrate that T1D responders to WP carried HLA-DR4 in 95% of cases, a frequency that was significantly higher compared with non-responders (Table 3.4 B). In addition, monoclonal anti-DR antibodies blocked T cell proliferation and Th1 cytokine response to wheat protein in T1D patients (Figure 3.8 and 3.9).

T1D patients develop coeliac disease with a frequency that is 5-25 times the frequency of CD in the general population [83,84,173]. Patients with T1D and coeliac disease share some HLA-associated risk genes such as HLA-DQ2 and HLA-DQ8 (HLA-DQB1*0302). The frequency of HLA risk genes is different comparing the two diseases: 90-95% of coeliac patients are HLA-DQ2 and 5-10% are HLA-DQ8 [174] in contrast up to 50% of diabetes patients less than 5 years old are HLA-DQ8/DQ2 heterozygotes [175]. Some investigators have suggested that the immune response to wheat in T1D patients is simply a reflection of shared genetic risk. Our data do not support this proposal. The frequency of HLA-DQB1*02 was not significantly different between T1D patients and controls (p=0.6; Table 3.3 A) and most importantly, we did not detect any significant differences for HLA-DQB1*02 between responders and non-responders (P=0.75) (Table 3.4 B). We also evaluated the HLA-DR and -DQ haplotypes by high resolution typing in T1D patients (Figure 3.7). By using this analysis we hoped to be able to discriminate WP responders from non-responders. Surprisingly, we did not find a specific pattern to differentiate responders from non-responders. In general, a positive response to WP in patients was more frequently associated with the HLA-DR4 (DRB1*0401/4) DQB1*0301/2 haplotypes and it was not associated with HLA-DR3/DQB1*0201 (Figure 3.7 A and B).

There are several reports indicating that the gut is mildly inflamed and unusually permeable to lumen antigens in diabetes-prone BBdp rats [101-103,144,167,176] and in patients with T1D [97,98,100]. Consistent with this, we determined that T1D patients also have an abnormal T cell response to other dietary antigens including OVA, gliadin and the gliadin 33-mer peptide (Figure 3.3). In addition, T cell responses to WP and OVA or gliadin 33-mer were closely correlated in T1D patients (Figure 3.4 C, D and E). By contrast, we did not find any significant differences between T1D and controls in response to TT, a recall antigen and

PHA, a positive control (Figure 3.3). Under normal conditions, the gut immune system suppresses immune reactions against dietary antigens or commensal bacteria, a process called oral tolerance [177]. Entry of an excessive amount of dietary antigens via a leaky gut barrier as has been reported in some studies of T1D patients could cause an imbalance in the gut immune system resulting in a breakdown of tolerance.

These data are consistent with previous reports suggesting a role for wheat proteins in diabetes-prone animals and humans 29,34,144,178. Previous evidence linking the development of diabetes in humans to wheat relied mainly on epidemiological studies. The results from the current study indicate that more than 45% of T1D patients display an abnormal immune response to wheat proteins. The finding that WP-responders were predominantly HLA-DR4, and not HLA-DQ2, suggests the likelihood that this effect was diabetes-specific. In addition, the presence of an HLA-DR4-restricted Th1 cell mediated immune reaction to these dietary proteins in T1D patients provides new data in support of a possible link between the gut immune system, wheat proteins and diabetes.

Chapter Four

Expression and purification of WP5212, a Glb1 homologue

Introduction

A novel environmental candidate diabetes-related antigen WP5212, a homologue of a wheat storage globulin protein was identified in our laboratory by Dr. Amanda MacFarlane. This protein was identified by screening a wheat cDNA expression library using sera from diabetic BBdp rats. The sequence of this clone has 90% homology at the nucleotide level and 80% homology at the amino acid level with *Triticum aestivum* wheat storage globulin, Glb1 protein [86]. From here on, WP5212 protein will be referred to as Glb1. Antibody reactivity against this clone in diabetic BBdp rats was significantly higher compared with both asymptomatic and control animals. In addition, islet inflammation and damage was correlated with antibody reactivity in this animal model of autoimmune diabetes [86]. The Glb1 coding DNA fragment was previously cloned into the pET17b expression vector to express it as a His-tag fusion protein in a bacterial system. Unfortunately, transformation of *E. coli* with this expression vector resulted in slow growth and the level of protein expression was insufficient for purification using Ni-NTA agarose columns. To evaluate the potential role of Glb1 in diabetes pathogenesis, it was necessary to produce and purify large quantities of Glb1 protein. Therefore, we generated a recombinant baculovirus expressing His-Glb1 protein. Purified protein was later used in various assays to analyze T cell proliferation and cytokine production. This protein was also used to establish an ELISA assay to measure Glb1 antibody levels in serum.

Experimental design and results

The baculovirus gene expression method was used to produce recombinant Glb1 in insect cells. A BakPAK™ Baculovirus Expression system kit was purchased from Clontech

Laboratories, Inc (Version #PR95847, Meadow Circle, CA). Recombinant baculovirus expression vectors were constructed in two steps. First, the target gene WP5212 was inserted into multiple cloning sites of the transfer vector BacPAK9, which also contained a bacterial plasmid origin of replication and an antibiotic resistance gene. This vector is able to replicate in *E. coli*, but it is not able to replicate in insect cells. In the second step, the recombinant transfer vectors (BacPAK9-His-Glb1) and a viral expression vector (BacPAK6) were cotransfected into Sf21 insect cells. Double recombination between viral sequences in the transfer vector and the corresponding sequences in the viral DNA transferred the target gene to the viral genome.

pBacPAK9-WP5212 transfer vector construction

To construct a recombinant transfer vector for expression of Glb1, the His-Glb1 DNA fragment from the previously constructed pET17b-His-Glb1 [179] vector was inserted into the appropriate site of pBacPAK9. Briefly, pET17b-His-Glb1 and pBacPak9 were transformed into DH5α to produce a sufficient amount of plasmid DNA and His-Glb1 insert for cloning. Both vectors were purified and digested with *Xba* I and *Xho* I (Figure 4.1 A). Digested plasmid DNA from pBacPAK9 and the His-Glb1 fragment from pET17b-HIS-Glb1 vector was isolated from agarose gels and purified using a QIAEX II gel extraction kit (Cat#20021, Mississauga, ON) (Figure 4.2 A-C). His-Glb1 gene was cloned into the *Xba* I and *Xho* I restriction enzyme site of the baculovirus transfer vector pBacPak9 following instructions provided with T4 ligase (Invitrogen, Carlsbad, CA). Transformation was performed using ligated DNA from the previous step. Three colonies were grown after an overnight incubation at 37°C (Figure 4.2 D). To confirm the presence of the insert (His-Glb1) within the plasmid DNA of the three clones, plasmid DNA was purified using Wizard

plus SC minipreps (Promega, Cat#A1330, Madison, WI) and digested with *Nco* I or *Xho* I (Figure 4.2 C). Sequencing performed at the University of Ottawa Biotechnology Institute confirmed that the vector contained the His-Glb1 sequence.

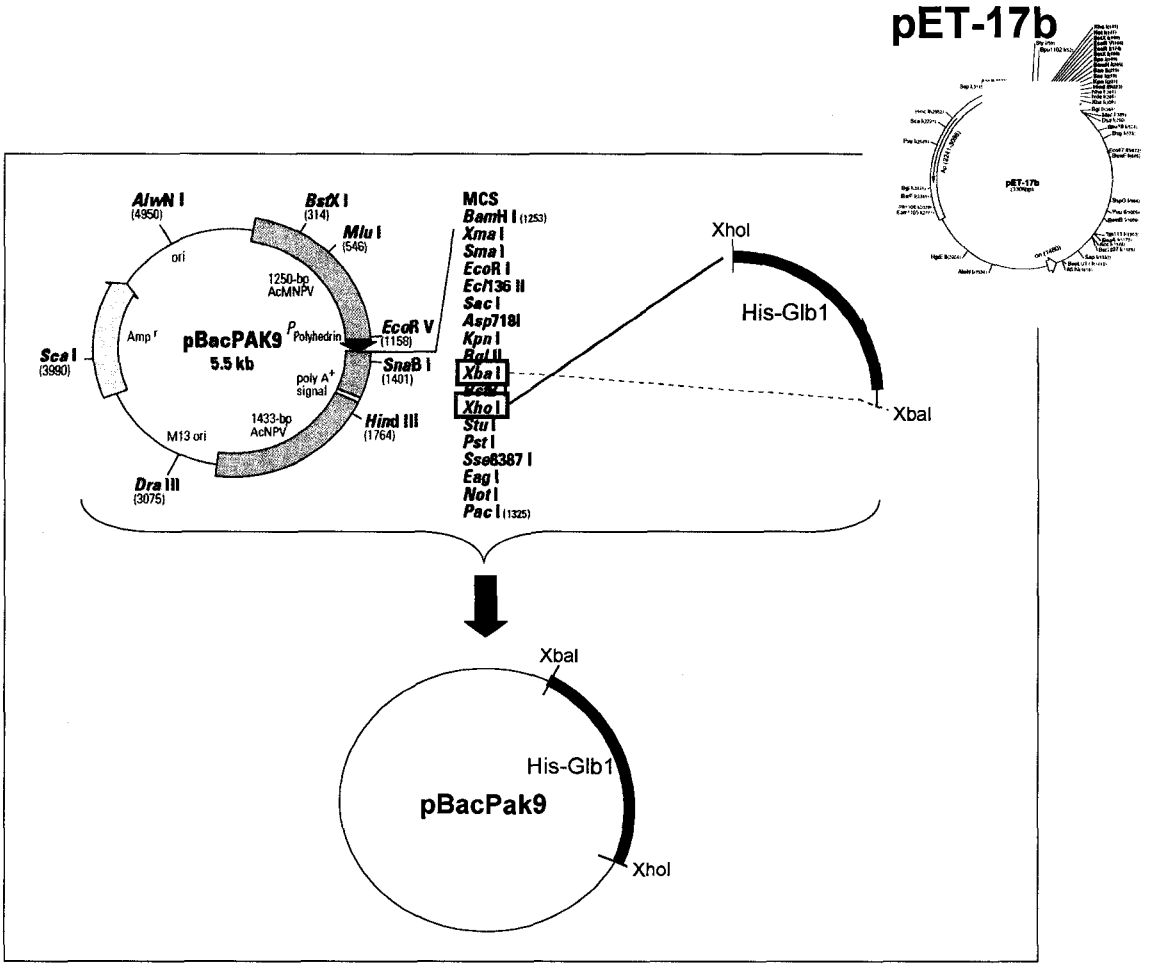
Construction of a pBacPak6-His-Glb1 recombinant viral expression vector

The His-Glb1 gene was transferred into the baculovirus DNA by forced recombination between the transfer vector and BacPAK6 viral DNA (Figure 4.1 B). *In vivo* homologous recombination between the plasmid and viral DNA rescues the viral DNA and transfers the target gene to the viral genome [180]. Briefly, appropriate concentrations of pBacPAK9-His-Glb1 plasmid DNA and BacPAK6 viral DNA were incubated with Bacfectin according to the manufacturer's instructions (Clontech Laboratories, Meadow Circle, CA). The Bacfectin-DNA mixture was added to monolayers of SF21 insect cells in 6 well plates. After 3 days, the culture supernatant was harvested and used as primary stock of recombinant virus.

Figure 4.1 Construction of BacPak9-His-Glb1 transfer vector and recombinant BacPak6 His-Glb1 baculovirus expression vector

(A) The His-tagged Glb1 nucleotide sequence was purified after digestion of the pET17b-His-Glb1 expression vector with XhoI/XbaI restriction enzymes. Next, the His-Glb1 fragment was subcloned into the XhoI/XbaI restriction enzyme sites of the BacPak9 transfer vector (BD Bioscience). **(B)** Transfer of the His-Glb1 gene to the baculovirus expression vector by forced recombination between a transfer vector and BacPAK6 viral DNA.

(A)


pET-17b
AlwN I (4950)
BstX I (314)
Mlu I (546)
ori
1250-bp AcMNPV
Amp r
pBacPAK9
5.5 kb
P Polyhedrin
poly A+ signal
1433-bp AcNPV
M13 ori
Dra III (3075)
Sca I (3990)
EcoR V (1158)
SnaB I (1401)
Hind III (1764)
MCS
BamH I (1253)
Xma I
Sma I
EcoR I
Ecl136 II
Sac I
Asp718I
Kpn I
Bgl II
Xba I
Xho I
Stu I
Pst I
Sse8387 I
Eag I
Not I
Pac I (1325)
XhoI
His-Glb1
XbaI
XbaI
His-Glb1
pBacPak9
XhoI

(B)

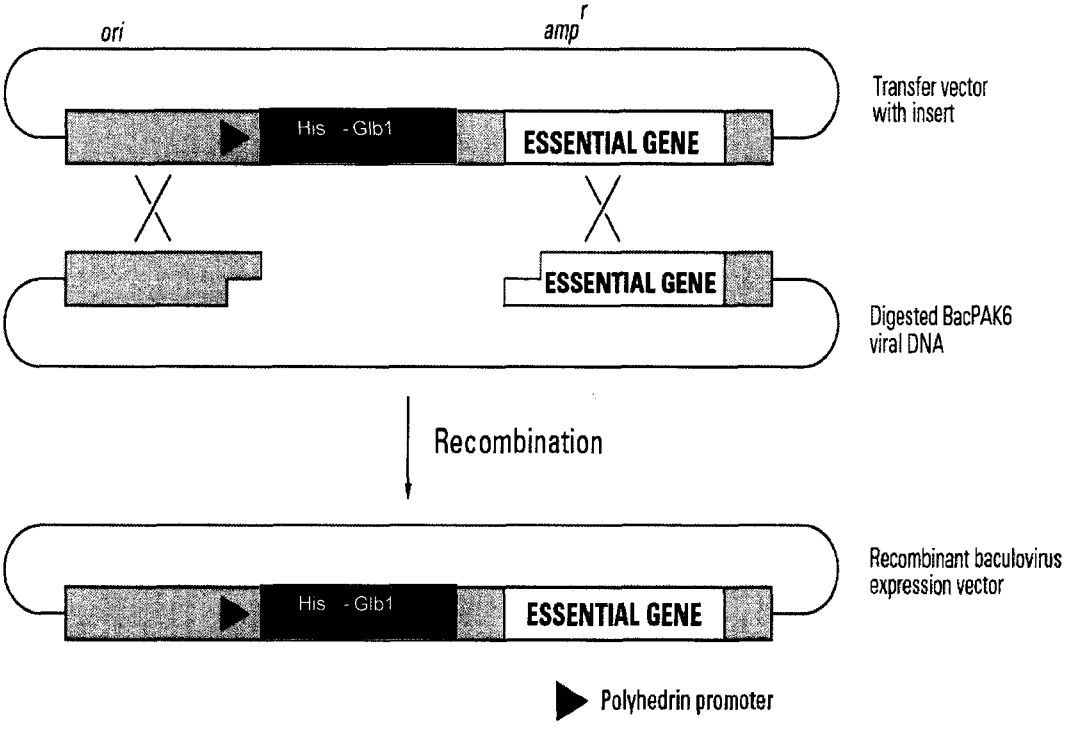

ori
amp r
His - Glb1
ESSENTIAL GENE
Transfer vector with insert
ESSENTIAL GENE
Digested BacPAK6 viral DNA
Recombination
Recombinant baculovirus expression vector
His - Glb1
ESSENTIAL GENE
Polyhedrin promoter

Figure 4.2 Amplification, purification, and isolation of BacPak9 His-Glb1 transfer vector

Panel A, pET17b-His-Glb1 and pBacPAK9 before and after digestion with Xba I and Xho I (lane 1, 1 kb plus DNA ladder; lane 2-3, pET17b-His-Glb1 plasmid before and after digestion; lane 4-5, pBacPAK9 plasmid before and after digestion; Lane 6, super coil ladder. **Panel B,** Isolation and purification of His-Glb1 insert from digested pET17b-His-Glb1 plasmid and purification of digested pBacPAK9 *a)* lane 1, 1 kb plus DNA ladder; lane 2-3, empty well; lane 4-10, plasmid after digestion; Lane 11, empty well; lane 12, digested pBacPAK9 *b)* gel from well number 4-10 for purification of insert and well number 12 for pBacPAK9 purification. **Panel C,** Confirmation after gel purification of digested His-Glb1 insert and pBacPAK9 (lane 1, 1 kb plus DNA ladder; lane 2, digested and gel purified pBacPAK9; lane 3, empty well; Lane 4, insert after gel purification; lane 5, purified pET17b-His-Glb1 before digestion. **Panel D,** cloning His-Glb1 gene into baculovirus expression vector pBacPak9 with T4 ligase (Invitrogen) (lane 1, 1 kb plus DNA ladder; lane 2, pBacPAK9 plasmid; lane 3-5, pBacPAK9-His-Glb1 plasmid isolated from colony 1-3; lane 6, 1 kb plus DNA ladder).

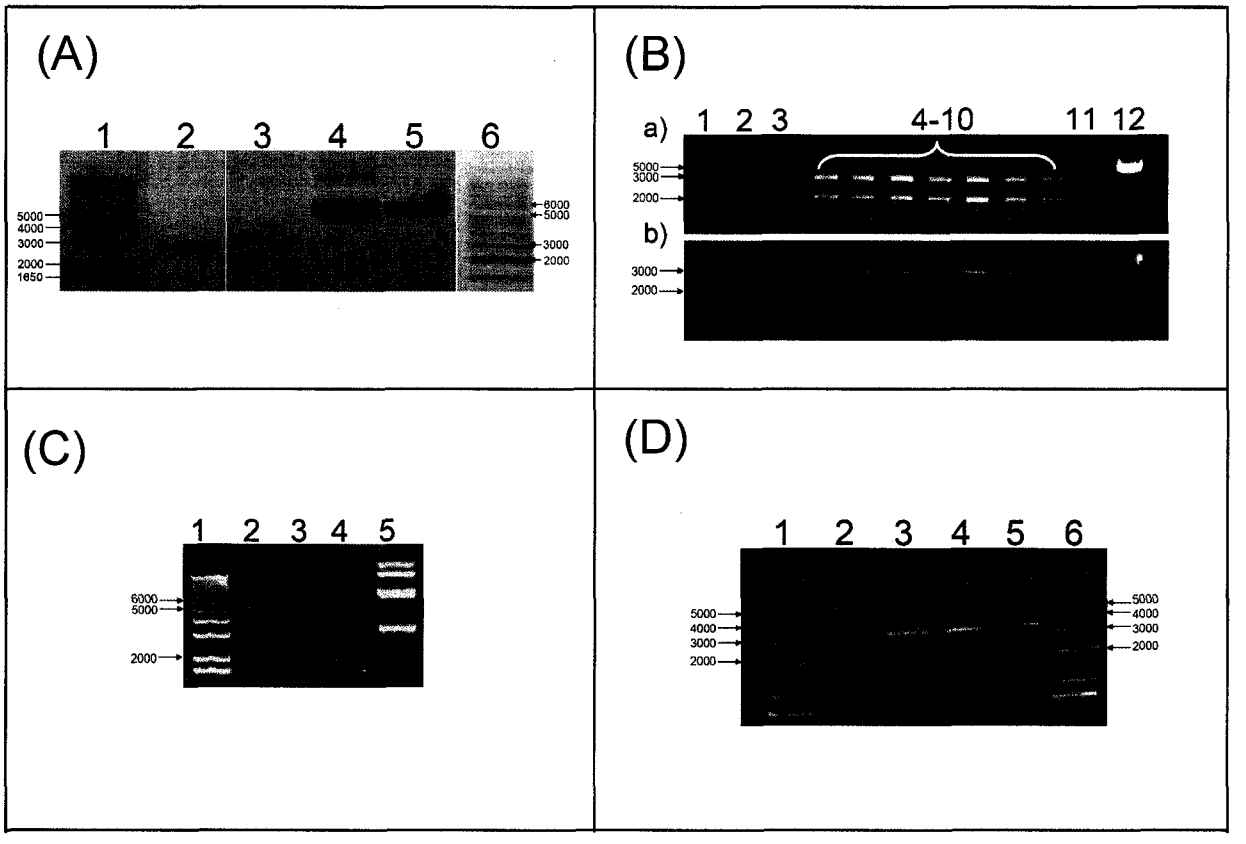
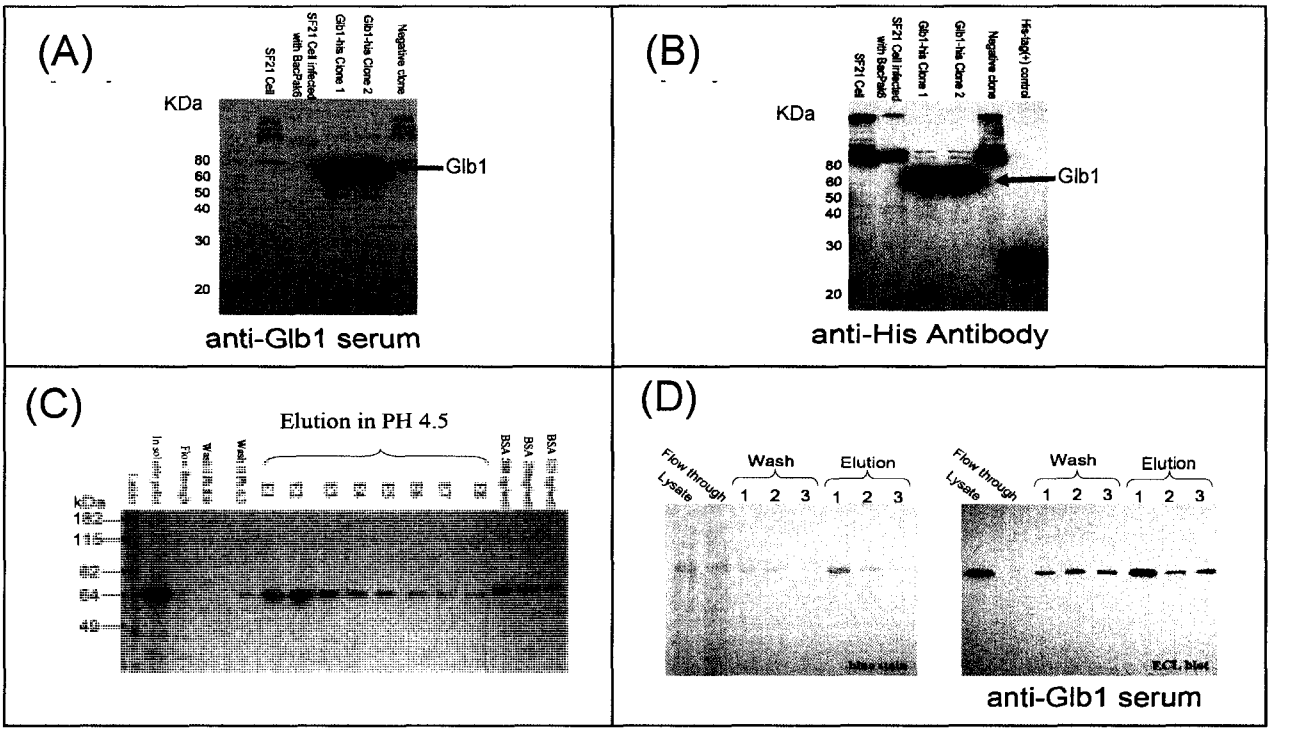

(A)
1 2 3 4 5 6
5000
4000
3000
2000
1650
6000
5000
3000
2000
(B)
a)
1 2 3
4-10
11 12
5000
3000
2000
b)
3000
2000
(C)
1 2 3 4 5
6000
5000
2000
(D)
1 2 3 4 5 6
5000
4000
3000
2000
5000
4000
3000
2000

Figure 4.3 Expression and purification of recombinant His-Glb1 protein.

Panel A and B, Analysis of recombinant His-tag Glb1 expression with anti-Glb1 serum and anti-His antibody. SF21 cell lysate was electrophoresed on a 12% SDS-Page gel, transferred, and subjected to western blotting with anti-Glb1 serum (*Panel A*) and anti-His antibody (*Panel B*). SF21 cell lysate and BacPak6-His-Glb1 infected SF21 cells lysate, and the His-tag (+) protein was used as controls. An arrow indicates the location of Glb1 protein and molecular mass standards are given in kiloDalton (kDa). **Panel C and D,** Analysis of recombinant His-Glb1 purification. Two techniques of Ni-NTA purification were used to purify lysate containing the His-Glb1. The insoluble form of Glb1 was extracted using 8M urea and eluted by changing the pH (*Panel C*). The soluble form of Glb1 was extracted by lysing of SF21 cells and eluting the protein using a high concentration of imidazole (*Panel D*).


(A)
SF21 Cell
SF21 Cell infected with BacPak6
Glb1-his Clone 1
Glb1-his Clone 2
Negative clone
KDa
80
60
50
40
30
20
Glb1
anti-Glb1 serum
(B)
SF21 Cell
SF21 Cell infected with BacPak6
Glb1-his Clone 1
Glb1-his Clone 2
Negative clone
His-tag(+) control
KDa
80
60
50
40
30
20
Glb1
anti-His Antibody
(C)
Elution in PH 4.5
Lysate
Insoluble pellet
Flow through
Wash I
Wash II
E1
E2
E3
E4
E5
E6
E7
E8
BSA 500 ug loaded
BSA 250 ug loaded
BSA 125 ug loaded
kDa
182
115
82
64
49
(D)
Flow through
Lysate
Wash
1 2 3
Elution
1 2 3
Flow through
Lysate
Wash
1 2 3
Elution
1 2 3
anti-Glb1 serum

Isolating recombinant virus and preparation of passage one virus stock

Most viruses in the primary stock were recombinant, but this stock was not pure. To obtain a pure clone of the recombinant virus, plaque assays were used to produce individual plaques. Briefly, the manufacturer's instructions were followed to obtain a single plaque from the primary stock. Two individual plaques were obtained and viruses released from those plaques were used to infect a dish containing SF21 cells. After 3-4 days of culture, supernatants were harvested, placed in sterile centrifuge tubes and used as the passage one virus stock. After removing medium from the virus-infected cell cultures, cells were harvested for Glb1 expression analyses. Western blot analyses using anti-Glb1 serum and anti-His antibody confirmed that both isolated clones expressed a 62-63 kDa protein, which was the predicted size of His-Glb1 (Figure 4.3 A and B). For long term storage, one milliliter of passage one virus stock from each plaque was kept at -70°C.

Preparation of passage two virus stock

Passage one virus stocks were titrated using standard protocols, as described in the manufacturer's instructions. Following this, the passage two virus stocks were prepared. Briefly, 50 ml of SF21 cell suspension culture with a density of 4×10^5 cells/ml were infected with the passage one virus stock (Multiplicity of infection, M.O.I.= 0.1). After 4 days of culture at 27°C, suspensions were centrifuged at 1000×g for 5 min to remove cells and debris. Culture supernatants were transferred to fresh sterile tubes, stored at -70°C, and labeled as the passage two virus stocks. Virus titration was determined in both passage two virus stocks and was 2.6×10^8 and 1.5×10^8 PFU/ml in plaque 1 and 2 respectively.

Large scale His-Glb1 production in SF21 insect cells

To prepare suspension cultures for large-scale protein production, 150 ml suspension cultures were seeded with 2×10^5 SF21 cells /ml. When concentration of the cells reached 10^6 cells /ml, the culture was infected with recombinant virus stock to reach an M.O.I. of 10. To achieve maximum protein expression, we used BacPAK Complete Medium and cells were infected when they reached log phase. After 2 days of incubation at 27°C, cells were harvested and centrifuged in 50 ml sterile tubes at 1000×g for 10 min, the supernatant was discarded and the cell pellet was stored at -20°C.

Purification of His-tagged Glb1 from Baculovirus-infected SF21 cells

To test the status of the expressed protein, soluble and insoluble fractions of the pellet were evaluated using SDS-PAGE. Briefly, the cell pellet from a 50 ml tube was suspended in 20 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, protease inhibitor tablet, 1 mM PMSF, and 10 μM β-mercaptoethanol at pH 8.0) and ultrasonicated at 4°C. Lysed cells were centrifuged at 3500×g for 10–30 min at 4°C. The supernatant was collected as a soluble fraction and the remaining cell pellet (insoluble fraction) was suspended separately in 10 ml of lysis buffer containing 8M Urea. Aliquots from each fraction were analyzed using SDS-PAGE followed by staining with blue stain. His-Glb1 was present in both soluble and insoluble fractions (Figure 4.3 C and D). The recombinant His-Glb1 was purified from both soluble and insoluble fractions using Ni-Sepharose beads (Figure 4.3 C and D). Two different strategies were used to elute proteins from the beads. An imidazole technique was used for the soluble fraction. In this technique, lysis and column wash buffers (pH 8.0) contain a low concentration of imidazole (10 mM and 20 mM, respectively), which

competes with the histidine of proteins so there is less non-specific binding to the column. In the elution buffer, the imidazole concentration is raised to 250 mM, which is enough to compete for space in the nickel column and release recombinant 6x-His-proteins. An acid elution technique was used for the insoluble part of His-Glb1. Briefly, buffers containing 8 M urea were used at different pH for purification of the protein, pH 8.0 for loading the SF21 lysate on the nickel column, pH 6.3 for washing the column and pH 4.5 to elute the recombinant protein

Discussion

In order to produce pure recombinant His-Glb1 without LPS contamination on a large scale, we expressed this protein using a baculovirus/insect cell expression system. This system has the capacity to produce large quantities of recombinant protein with biological properties similar to the native protein [181]. The cDNA carrying the His-Glb1 gene was successfully cloned into a baculovirus expression vector. The soluble and insoluble form of His-Glb1 was expressed at high levels, and was efficiently produced in SF21 insect cells. The production of His-tagged fusion proteins enabled purification using immobilized metal affinity chromatography which is a rapid and a highly efficient method to purify proteins in their native form [182].

Previously in our laboratory His-Glb1 (WP5212) protein was cloned into a bacterial expression vector. However, *E. coli* containing the expression vector demonstrated slow growth and the level of protein expression was not sufficient for purification [179]. This is the first report of preparation of a highly purified His-Glb1 protein. This reagent was used to evaluate cellular immune responses and to develop a capture ELISA for evaluation of anti-Glb1 antibody in human sera.

It has been reported that C-terminal-His fusion proteins express considerably lower levels of protein compared with N-His fusion proteins due to conformational changes [183]. The evaluation of recombinant protein production levels indicated that our His-Glb1 protein containing the 6x-His tag at the N-terminal was expressed at a high level (Figure 4.3 A and B).

Two individual plaques were obtained after generation of recombinant viruses. We used both plaques to prepare passage two of the recombinant virus. The amount of protein expressed in both passages was high and similar in both clones (Figure 4.3 A and B). Importantly, this recombinant protein was specifically recognized by polyclonal sera derived from rabbits which were vaccinated with two peptides from Glb1 or sera from a highly wheat sensitive patient (Figure 4.3 A) and in addition was recognized by anti-His antibody (Figure 4.3 B). These data indicate that the presence of the His-tag did not influence the binding of anti-Glb1 antibodies generated in rabbit or human.

We used one of the virus stocks to produce bulk quantities of recombinant His-Glb1 protein. Glb1 fusion protein was over expressed in both cytoplasm (soluble form) and in organelles (insoluble form). We detected good amounts of protein in both fractions and we were able to purify these using Ni-sepharose. We successfully isolated His-Glb1 with high efficiency under denaturing conditions. However we were not able to avoid precipitation of Glb1 during dialyses in order to remove urea. Although Glb1 from the soluble part showed a somewhat lower level of purification compared with the insoluble form, we used the soluble fraction because of the easy purification and the benefit of using the soluble form of the protein in our future assays.

In summary, we were able to produce large quantities of Glb1 by infecting SF21 insect cells with recombinant pBacPak6-His-Glb1 virus. This protein was purified using the Ni-sepharose technique. The purified protein was used in a capture-ELISA for Glb1 antibodies and in cell proliferation assays to determine the T cell immune responses against this dietary protein.

Chapter Five

Immune reactivity to a Glb1 homologue in a highly wheat-sensitive patient with type 1 diabetes and coeliac disease

Introduction

T1D and CD are inflammatory disorders of autoimmune origin requiring genetic susceptibility and interaction with environmental factors [173]. The co-incidence of T1D and CD has been reported by several groups [84,173,184,185]. Although the pathogenesis of CD is unclear, development of disease depends on the presence of gluten in the diet in genetically predisposed individuals who display HLA-DQ2 in more than 95% of cases [126]. There is evidence that some cases of T1D could be wheat–related [80,87,88,173] . One to 6 percent of T1D patients develop CD, a rate that is 5 to 25 times the incidence of this disease in the general population [84,129-131,173,184]. Interestingly, in one study 23% of patients with CD developed islet related antibodies [134]. There are reports that 90% of cases with both diseases develop diabetes first suggesting that in cases who develop CD first, a gluten free diet has a protective effect and may prevent the development of diabetes [136]. Recently, a wheat protein, WP5212 was identified as the first diabetes-related wheat protein in diabetes-prone rats [86]. This protein has 80% homology at the amino acid level with wheat storage globulin-1 (Glb1).

We reported a case of a 28-year-old Caucasian woman with T1D and biopsy-proven coeliac disease, who presented with unusually high sensitivity to wheat proteins. She had a 10 year history of T1D and presented early in 1999 with onset of diarrhea followed by oral ulcerations and severe lip swelling. Below is the clinical history of this individual which was extracted from clinician reports and published by us recently [143]. "The diarrhea consisted of 4 to 10 bowel movements in a 24-hour period including nocturnal stools in this patient. There was no family history of bowel disease nor any joint, skin, or eye symptoms. At diagnosis, anti-gliadin IgG, tissue transglutaminase (7 U/ml) and endomysial antibodies

were positive. CD was confirmed with a duodenal biopsy showing mild villous atrophy despite 6 months of a standard gluten-free diet. Colonoscopy and small bowel follow-through were normal. Biopsy of the oral lesion showed non-caseating granulomas which were negative for IgG, IgA, IgM, C3, fibrinogen and albumin. Mycobacterium tuberculosis, fungus and other infectious agents were negative as well. The diarrhea and lip swelling improved initially with the gluten-free diet but the symptoms recurred within a few months with worsening of the oral ulcerations, lip swelling and continued diarrhea".

"In December 2001, the mucosa of her lower lip was extensively ulcerated with a fistula in the lower right lip area. The patient could only open her mouth 1.5 cm and both lower and upper lips were swollen. She was having 7-10 bowel movements in a 24 hour period with nocturnal incontinence. A magnetic resonance scan of her head and neck area did not show any areas of fibrosis therefore making the diagnosis of Melkersson-Rosenthal less likely. Following this, the patient initiated a strict specified carbohydrate, grain-free diet. In a period of weeks the diarrhea resolved with an average of 2 bowel movements a day and the healing of her mouth ulcerations. Over the next few months, her lips returned to a normal level with a scar and fibrosis in the area of the previous fistula".

Results

Subject

The case was a 28-year-old female with type 1 diabetes and CD (T1D/CD). The patient had both the diabetes associated HLA types (DRB1*0401, DRB1*0301 and DQB1*0302) and the coeliac-associated HLA types DRB1*0301, DQB1*0201, and HLA DR53. Eight subjects with no significant medical history and with an age range of 24-40 years were chosen as controls and investigated in parallel with the case. Analysis of the

patient's blood was performed on samples obtained on two occasions during clinical remission. The study was approved by the Ottawa Hospital Research Ethics Board and informed consent was obtained.

Antibody reactivity to Glb1

Clone WP5212 (Glb1)

Data from our case report[143] showed strong antibody reactivity to clone WP5212 (Glb1). In contrast, the controls did not show reactivity to this clone. Antibody reactivity to two non-diabetes-related wheat proteins from a cDNA library, WP12111 and WP23112, was low for both the patient and controls (Figure 5.1). Antibody reactivity was measured by immunoscreening of wheat clones with serum and evaluated using densitometry and was reported as mean intensity/pixel [179].

Purified recombinant Glb1 protein

After purification of recombinant Glb1, an ELISA was established to evaluate serum Glb1 antibody levels in patients and controls (see chapter 6). The serum Glb1 level in this patient with T1D/CD was high at all dilutions, the optical density (OD) of dilutions for this patient was 4-6 times higher compared with the mean OD in controls (n=8) (Figure 5.2).

Reactivity of PBMC to WP components

T cell proliferation assays were performed using a CFSE fluorescence-based technique. We stimulated fresh PBMC from this type 1 diabetes/coeliac disease patient and control subjects with a series of dietary, coeliac associated and control antigens. $CD3^+$ T-cells from the T1D/CD patient proliferated in response to different concentrations of an extract of partially digested wheat gluten proteins and purified gliadin. In contrast, the responses to a coeliac

related 33-mer peptide antigen, diabetes-related autoantigens, human GAD and insulin were low (data not shown). PBMC of the patient also proliferated in response to Glb1. Responses to another dietary antigen, ovalbumin, were low and similar to our control group. There was a strong response to the recall antigen, tetanus toxoid, as well as the mitogen, PHA in the patient compared with controls. To evaluate whether response to WP in this patient was associated with HLA-DR, as we observed in T1D patients, the effect of HLA-DR antibody was evaluated on response of T cells to WP. The addition of HLA-DR antibody blocked the $CD3^+$ T cell response to wheat protein (Figure 5.3 A). IFN-γ and IL-5 were measured in the supernatant of cultured PBMC from the patient (Figure 5.3 B). Both cytokines were secreted in the culture supernatants in response to WP, gliadin, Glb1 and TT and the concentration was much higher compared with the basal level detected in control wells containing PBMC in medium alone. T cells which proliferated in response to Glb1 secreted a higher amount of the Th1 cytokine, IFN-γ, relative to the Th2 cytokine, IL-5 (Figure 5.3 B).

Figure 5.1 Wheat-sensitive patient with Glb1 antibody reactivity

(A) A highly wheat-sensitive patient with type 1 diabetes and coeliac disease developed swelling of the lip and ulceration of the cheek, following a standard gluten–free coeliac diet. **(B)** There was strong antibody reactivity to the Glb1 clone WP5212 (pictured beside the graph) as further illustrated in the densitometric analysis (graph), black bars are patient results; open bars are age-matched healthy subjects (mean ± SD, n=8). Antibody reactivity = mean intensity/pixel x 10^3 expressed relative to a negative clone, WPCON.

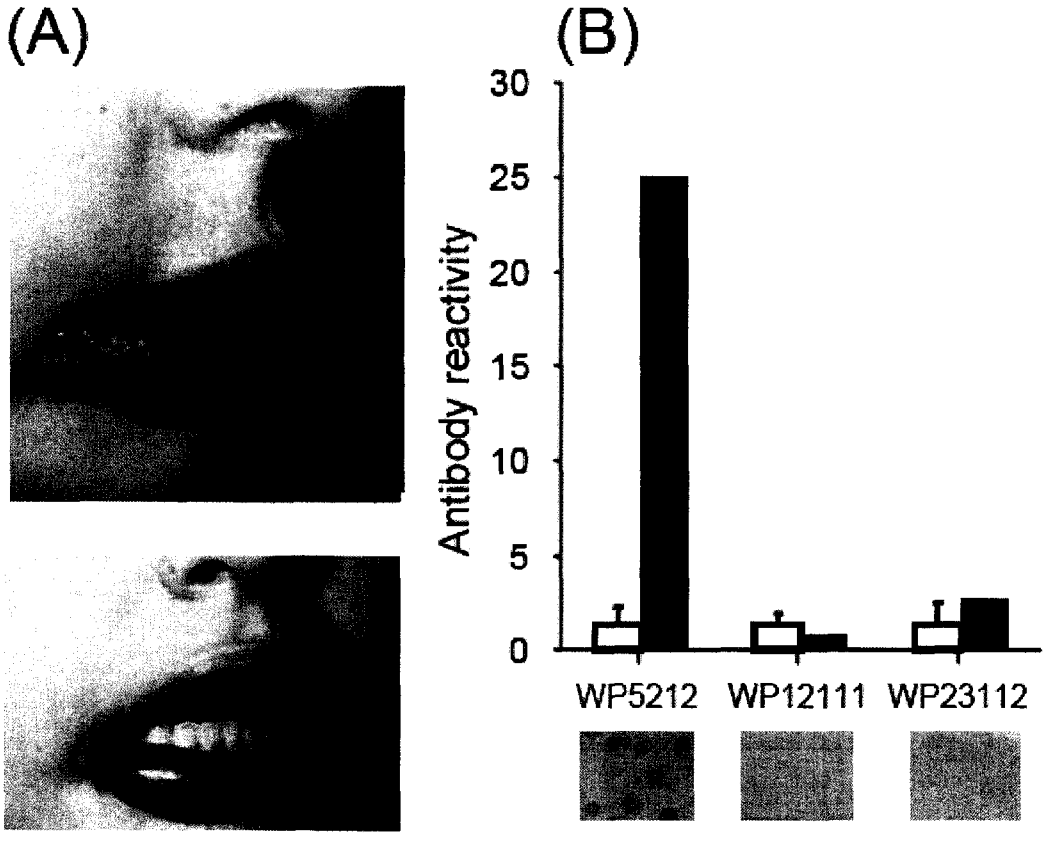


Figure 5.2 Serum Glb1 antibodies in a T1D/CD patient and controls determined using ELISA

Sera of the T1D/CD patient and healthy controls were diluted at different ratios and the absorbance in triplicate sample wells were measured at 450 nm.

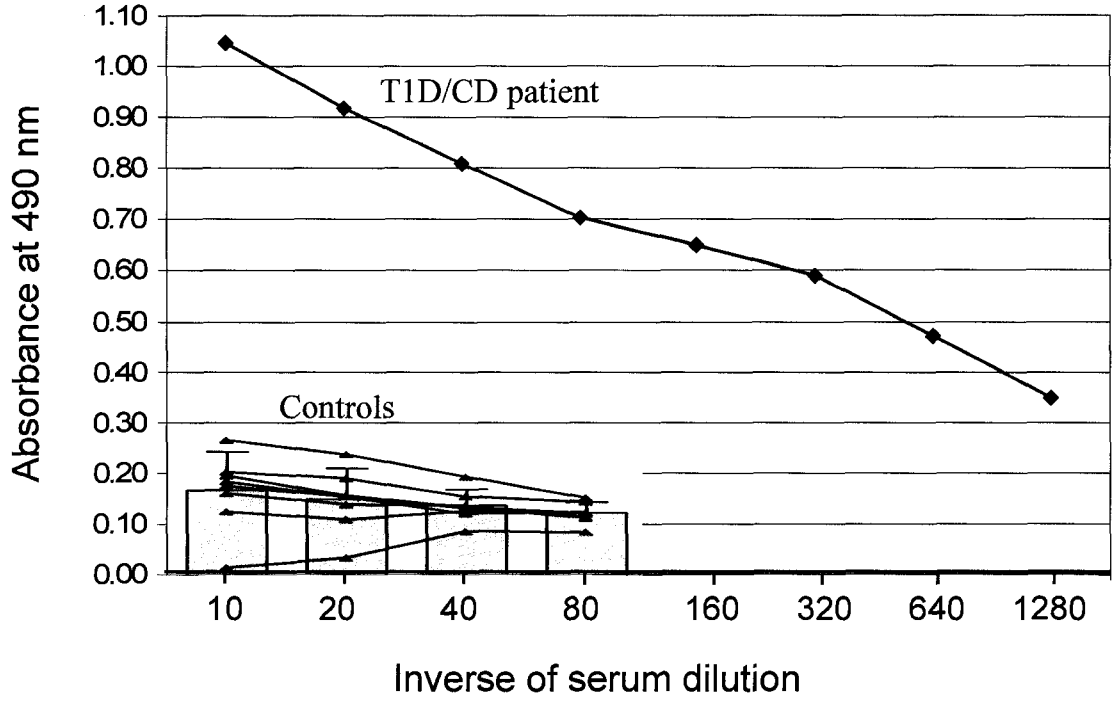
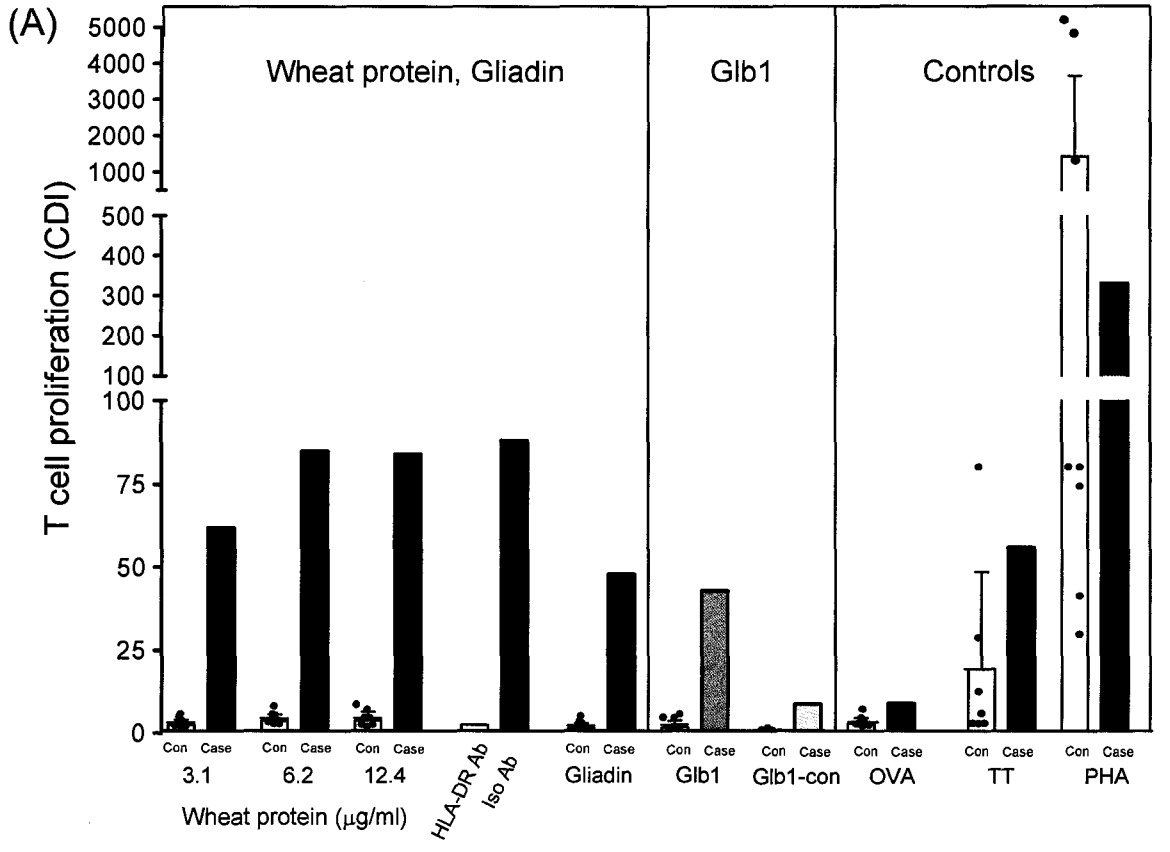
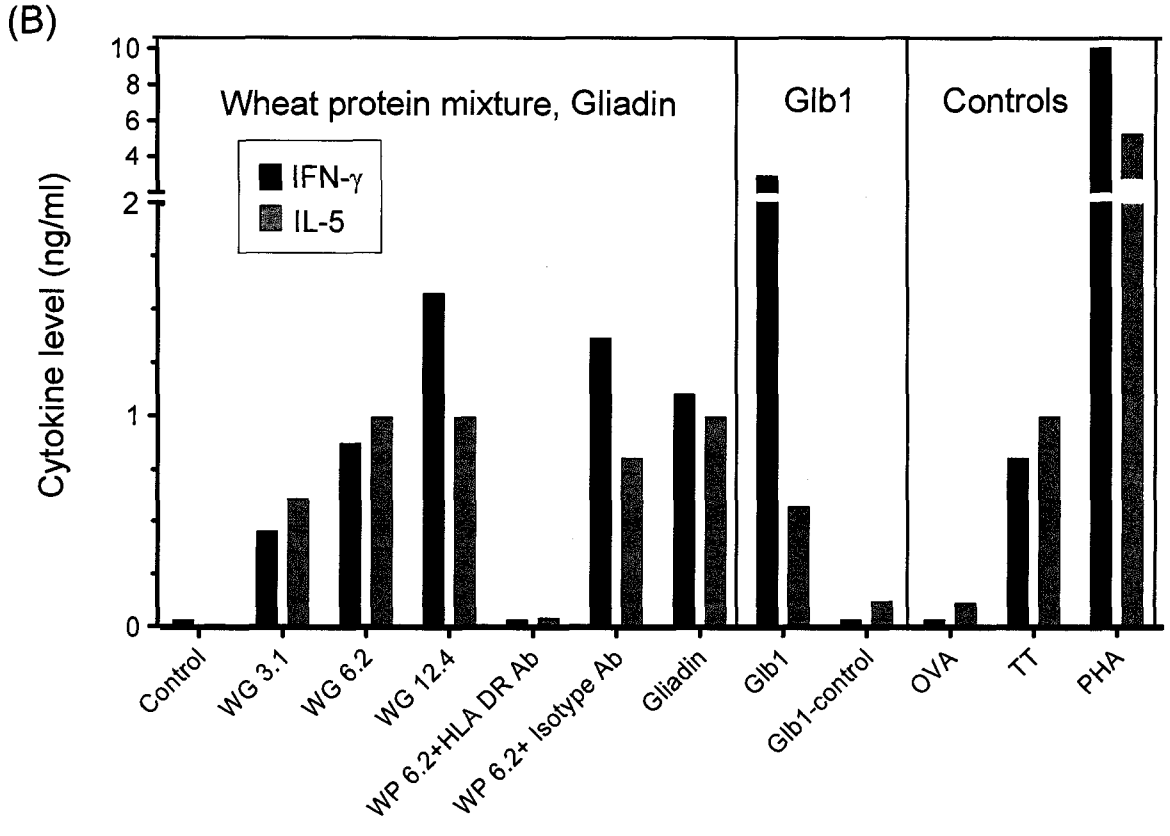

1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Absorbance at 490 nm
T1D/CD patient
Controls
10
20
40
80
160
320
640
1280
Inverse of serum dilution

Figure 5.3 Wheat protein-induced T-cell reactivity in a T1D/CD patient but not in controls

(A) $CD3^{+}$T cells from the T1D/CD patient (case) showed strong proliferation in response to different concentrations of WP and gliadin. WP response was blocked by adding antibodies against HLA-DR, but not by adding the control isotype (Iso) antibody. T cells also responded to Glb1 (10 μg/mL) but not to Glb1 control. There was no response to an irrelevant food antigen, OVA, whereas response to the positive control recall antigen, tetanus toxoid (TT) and the T cell mitogen, PHA, was strong. None of the controls (con) responded to WP, gliadin, Glb1 and OVA, however responses to TT and PHA were present in controls. **(B)** Cytokine response of the patient PBMC to different antigens measured by ELISA, both cytokines, IFN-γ and IL-5 were present in the supernatant of cell cultures, Glb1-stimulated PBMC of the T1D/CD patient showed a Th1 type response, with IFN-γ most abundant.


(A)
T cell proliferation (CDI)
5000
4000
3000
2000
1000
500
400
300
200
100
100
75
50
25
0
Wheat protein, Gliadin
Glb1
Controls
Con Case
3.1
6.2
12.4
HLA-DR Ab
Iso Ab
Gliadin
Glb1
Glb1-con
OVA
TT
PHA
Wheat protein (μg/ml)


(B)
Cytokine level (ng/ml)
10
8
6
4
2
1
0
Wheat protein mixture, Gliadin
Glb1
Controls
IFN-γ
IL-5
Control
WG 3.1
WG 6.2
WG 12.4
WP 6.2+HLA DR Ab
WP 6.2+ Isotype Ab
Gliadin
Glb1
Glb1-control
OVA
TT
PHA

Discussion

It is well known that wheat gluten, barley and rye glutelins are able to provoke an inflammatory process in the gut of genetically susceptible individuals that results in CD. However, the role of these dietary antigens in T1D pathogenesis is unclear. Several reports suggest a circumstantial link between CD and T1D and there is an unusually high coincidence of T1D and CD ranging from 0.6-16.4% [127-132]. In coincident T1D and CD, most of the cases develop diabetes first, as was the case in our T1D/CD patient [136]. One theory to explain the low proportion of T1D/CD patients who develop CD first is that avoidance of wheat in the diet could protect some individuals from developing diabetes [186]. However, Ludvigsson *et al.* did not find a significant difference in risk of subsequent type 1 diabetes between individuals with a diagnosis of coeliac disease at 0–2 years of age and those diagnosed after 2 years of age [187]. In general, immune reactivity to wheat protein decreases when coeliac patients are following a gluten-free diet and it appears again only after exposure to these proteins. The T1D/CD patient in our study showed unusually high sensitivity to cereals. However, even when she was on a standard gluten-free diet for two years, the mucosa of her lower lip was ulcerated and a fistula formed through the lower right lip area (Figure 5.1 A). She suffered from inflammation in both lower and upper lip, and increased number of bowel movements. Clinical and pathological evaluation ruled out the possibility of Melkersson-Rosenthal syndrome and infectious diseases. The patient initiated on her own a specific-carbohydrate and cereal-free diet, promoted by E. Gottschall in the book "Breaking the vicious Cycle: Intestinal Health Through Diet" [188]. In a short period of time most of her clinical problems decreased and after a few months, her lips returned to normal with a scar and fibrosis in the area of the fistula. However, despite being on a strict

diet for more than 2 years and absence of clinical symptoms, her immune response to wheat proteins persisted and we were able to characterize a strong T cell response to WP and Glb1 [143]. In addition, she displayed a strong antibody response to Glb1 evaluated by ELISA.

PBMCs from healthy controls responded only weakly to wheat gluten proteins, Glb1 and the other antigens, but responses to the recall antigen, TT and the mitogen, PHA showed normal distribution in controls (Figure 5.3). Evaluation of cytokines in the culture supernatants indicated a Th1-biased phenotype specifically in response to Glb1. Dr. H. Chakir also confirmed at the transcriptional level using RT-PCR or gene microarray, a Th1-biased phenotype [143], characterized by increased expression of IFN-γ, TNF-α and IL-12Rβ2 mRNA. The T cells that responded to wheat protein were generally $CD4^+$ T cells (~95%) with a low percentage of $CD8^+$ T cells (~3%)[143]. T cell responses to WP's were blocked using HLA-DR antibody (Figure 5.3 A), indicating that the wheat protein peptides were mainly presented to T cells on MHC class II HLA-DR.

Identification of an abnormal immune response to Glb1 in this patient raises the question whether this response is associated with one disease or the other, T1D vs. CD. However, globulins including Glb1 are not generally considered to be coeliac antigens [189]. Our results, reported in this thesis indicate there is a subset of patients with T1D that display immune reactivity to wheat proteins including Glb1. Furthermore, data from chapter 6 of this thesis, report that Glb1 antibody levels were significantly higher in cases of young subjects who develop coeliac disease autoimmunity and are tissue transglutaminase positive (tTG^+) compared with high genetic risk controls.

In the present chapter we report the first indication of strong immune responses against Glb1 in a T1D/CD patient, who also showed unusually high immune response to

wheat. This finding made it essential to further differentiate whether responses to Glb1 were increased in patients with T1D alone or were linked somehow to coeliac disease. The possible role of Glb1 in the pathogenesis of both T1D and CD remains to be determined. Also it will be important to determine whether reactivity to Glb1 is a marker of T1D development in patients who are at risk for both diseases.

Chapter Six

Immune reactivity against Glb1 in young adult T1D patients and children with T1D or CD autoimmunity

Introduction

Food components, the exact nature of which remain unclear, are considered as environmental agents that influence the incidence of islet cell autoimmunity and T1D 21,79. To further study recombinant Glb1 protein, we analyzed cellular and humoral immune responses to Glb1 in patients with T1D. We hypothesized that a sub-population of individuals with T1D could have abnormal immune responses to Glb1. The main objectives in this part of the study were (1) to determine whether T cell immune responses to Glb1 occur in young adult patients with T1D, (2) to develop an ELISA to measure Glb1 antibody in serum, (3) to identify Glb1 humoral immune responses in young adult T1D patients and (4) to investigate whether these immune responses are present in young children with T1D or CD autoimmunity (presence of autoantibodies).

Experimental design

Immune reactivity to Glb1 was evaluated in three groups of samples: (1) a subset of young adults with T1D of long duration (Chapter 3, Table 3.1). Glb1 T cell responses were evaluated using the CFSE proliferation assay. Th1 and Th2 cytokines were analyzed using cytometric bead arrays, and Glb1 antibodies were evaluated by ELISA, (2) a selection of serum samples from children participating in the DAISY study who developed T1D islet autoantibodies were evaluated for Glb1 antibody level, and (3) a selection of serum samples from children participating in the CEDAR study, who developed coeliac autoimmunity were also evaluated for Glb1 antibody concentration.

Group 1 Young adult T1D patients

In a subset of T1D patients and healthy control subjects described in Chapter 3, we evaluated the response of peripheral blood T cells to recombinant Glb1 protein. T cell response was measured using the CFSE proliferation assay and reported as cell division index. A concentration of 10 μg/ml recombinant Glb1 was chosen from dose response studies for use in the proliferation assay.

Results

T cell reactivity to Glb1 in PBMC from T1D patients and controls

Of the 24 patients with T1D that I evaluated 10 (41%) had a positive proliferation response to Glb1 using the mean + 3 SD of CDI (CDI $\geq$ 9.3) in the control group as the cutoff (Table 3.1, Figure 6.1, p=0.005, Mann-Whitney U-test). The CDI in response to Glb1 was significantly different between patients (n=24, mean CDI= 14.9 ± 19.1, SD) and controls (n=8, mean CDI= 2.5 ± 2.2); the median (range) was 5.8 (1.0-70.0) for the patients and 2.3 (0.2-5.5) for the controls. T cell response to Glb1 did not correlate with age or duration of disease (data not shown).

T cell responses to Glb1 inhibited by monoclonal anti-DR antibodies

The number of patients in this part of study was not sufficient to enable us to compare the frequency of various HLA haplotypes in responders and non-responders. Therefore, to understand whether T cell responses to Glb1 protein were associated with HLA-DR, the blocking effect of anti-HLA-DR mAb on PBMC of 11 T1D patients was evaluated. Monoclonal anti-DR antibodies blocked T cell responses to Glb1 in responder T1D patients (Figure 6.1 B).

Immune response to Glb1 recombinant protein is not due to LPS contamination

To evaluate the possible contamination of Glb1 recombinant protein with LPS like molecules, endotoxin levels in the protein solution were measured (Table 3.4). The amount of LPS (0.0029 ng/μg of Glb1) was much lower than the amount required to stimulate T cells (Chapter 3, Table 3.3)[152]. To further confirm this, Polymyxin-B (PmB) which neutralizes LPS, was used to rule out the possible effect of LPS contamination in our T cell assay. PMB had no effect on T cell responses to Glb1, whereas PmB inhibited the T cell response to LPS (Figure 6.2; and also Figure 3.6 in chapter 3 contains the LPS Control).

Coincidence of $CD3^+$ T cell responses to WP and Glb1 in young adult T1D patients

There was a positive correlation between T cell responses to Glb1 and WP (Pearson's r correlation, r=0.49, p=0.016). Seven out of 9 T1D patients who had a positive proliferative response to WP also showed positive responses to Glb1. In contrast, nine individuals displayed T cell responses to WP, whereas two patients only displayed positive values for Glb1 T cell responses but not for WP (Figure 6.3).

IFN-γ and IL-6 are the dominant cytokines in the supernatant of Glb1 stimulated PBMC

The supernatants of cultures from nine T1D and seven control individuals were evaluated for cytokine secretion by Glb1-stimulated PBMC using flow cytometric bead arrays (CBA, Human Th1/Th2 cytokine kit II, Cat No. 551809, BD Biosciences, San Diego, CA). Some individuals with high CDI in response to Glb1 also produced high levels of cytokines (IFN-γ, IL-4, TNF and IL-6). Both Th1 and Th2 cytokines were present in the supernatants of cultures in response to Glb1 protein. However, the concentration of IFN-

γ and IL-6 in supernatants of some Glb1 responders was much higher ~ 10-20 fold more than that of IL-4 in the same individuals, for example, patients number 1, 3 and 4 (Figure 6.4).

Establishing a Glb1 ELISA assay using purified recombinant Glb1

A capture ELISA was established using purified Glb1 to measure IgG-Glb1 antibodies in serum of patients and controls. We reduced the background and improved the sensitivity of the assay by modification of two steps of the capture ELISA protocol. In the blocking step, human serum albumin was used to prevent non-specific binding of sera to the plate. The second modification was absorption of the secondary antibody with Glb1 to reduce nonspecific binding. For comparison between assays, Glb1 antibody levels were reported as arbitrary units. Arbitrary units were calculated using the OD of a reference patient serum included in each assay. The value of the reference serum was considered as 100 arbitrary units and the values of the other samples were calculated based on this sample. Two other sera with low and intermediate absorbance were used as internal controls in each assay. To test reproducibility of the Glb1 ELISA assay, within-assay precision was evaluated (Table 6.1). For a description of the assay refer to material and methods.

Figure 6.1 T cell responses to Glb1 homologue in T1D patients blocked by anti HLA-DR

(A) Response to purified Glb1 in T cells from individual donors evaluated using a CFSE proliferation assay. The Cell Division Index to Glb1 in patients with T1D (n=24) and healthy control subjects (n=8) is shown. T1D patients showed a higher mean CDI in response to Glb1 compared with controls. The mean + 3 SD (CDI > 9.3) was used as a cutoff for positive proliferative response. 40 % of patients were classified as Glb1 responders. **(B)** Monoclonal anti-DR antibody (5 μg/ml) blocked Glb1-induced T cell response in most of the Glb1 responders.

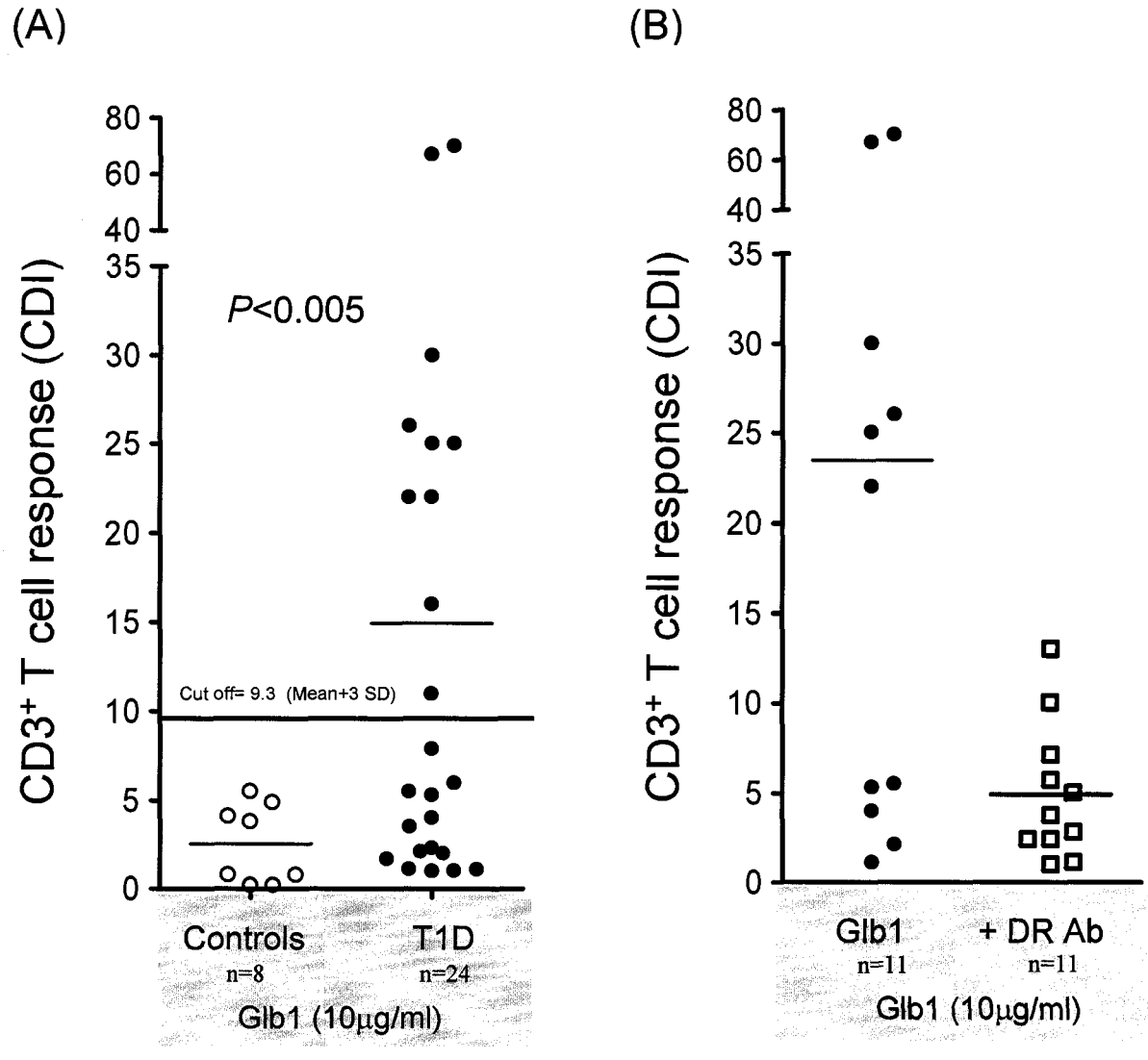
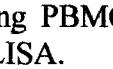

(A)
(B)
CD3+ T cell response (CDI)
80
60
40
35
30
25
20
15
10
5
0
P<0.005
Cut off= 9.3 (Mean+3 SD)
Controls
n=8
T1D
n=24
Glb1 (10μg/ml)
Glb1
n=11
+ DR Ab
n=11
Glb1 (10μg/ml)

Figure 6.2 Polymyxin B did not inhibit Glb1 induced T cell response

Response to Glb1 20 μg/ml and inhibitory effect of PmB 10 μg/ml on this response was evaluated using a CFSE proliferation assay in one individual (). PmB was added to wells 30 min before adding PBMC. IFN-γ () and IL-5 () were measured in culture supernatant using ELISA.

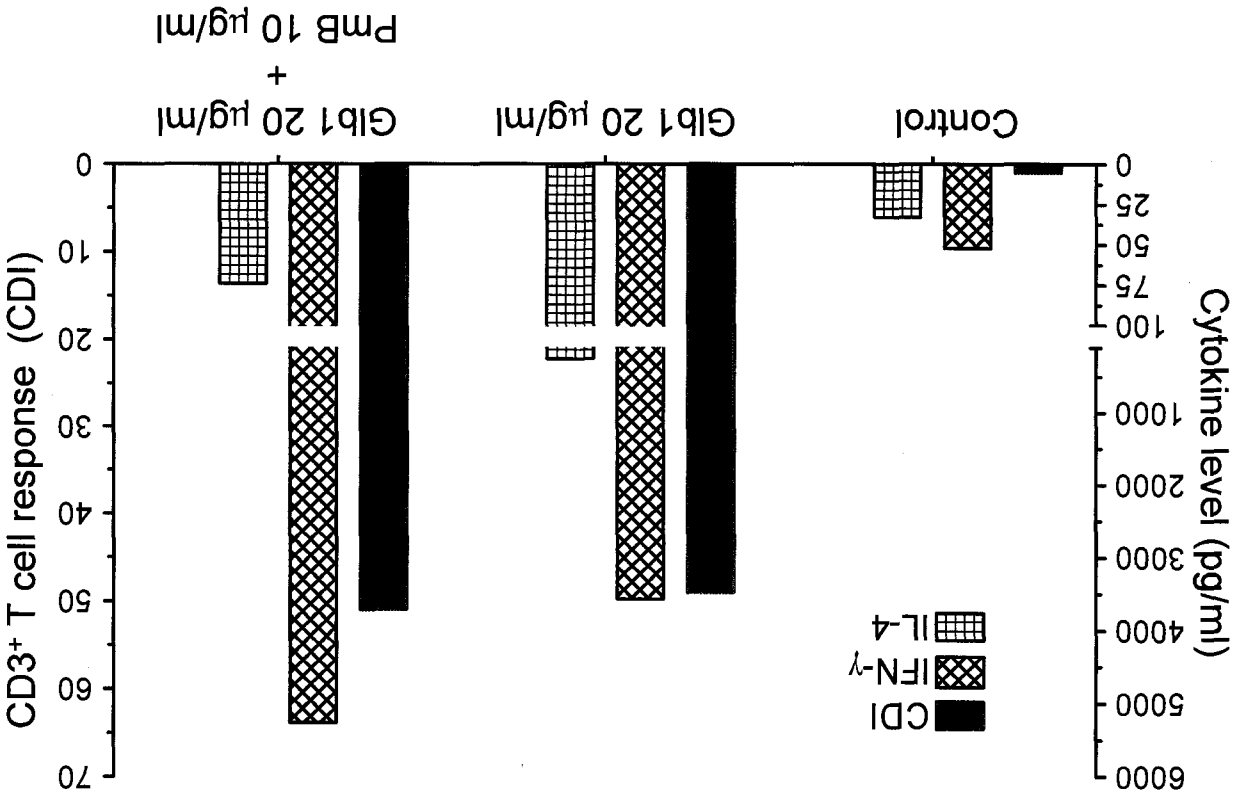

CD3+ T cell response (CDI)
0
10
20
30
40
50
60
70
Cytokine level (pg/ml)
6000
5000
4000
3000
2000
1000
100
75
50
25
0
CDI
IFN-γ
IL-4
Control
Glb1 20 μg/ml
Glb1 20 μg/ml
+
PmB 10 μg/ml

Figure 6.3 Coincidence of $CD3^+$ T cell responses to WP and Glb1 in T1D patients

A CFSE assay was used to evaluate T cell proliferation in response to either WP or Glb1 in 23 patients. Glb1 T cell responses were correlated with WP T cell responses ($r=0.496$, $p=0.016$, Pearson's r). Dashed lines show the cutoff value for WP and Glb1 T cell responses. **(A)** Results showed that 7 out of 23 T1D patients (red) displayed T cell responses to both WP and Glb1. **(B)** nine individuals (green) showed high WP T cell responses, without positive Glb1 T cell responses. In contrast two individuals only displayed positive values for Glb1 T cell response but not WP T cell responses.

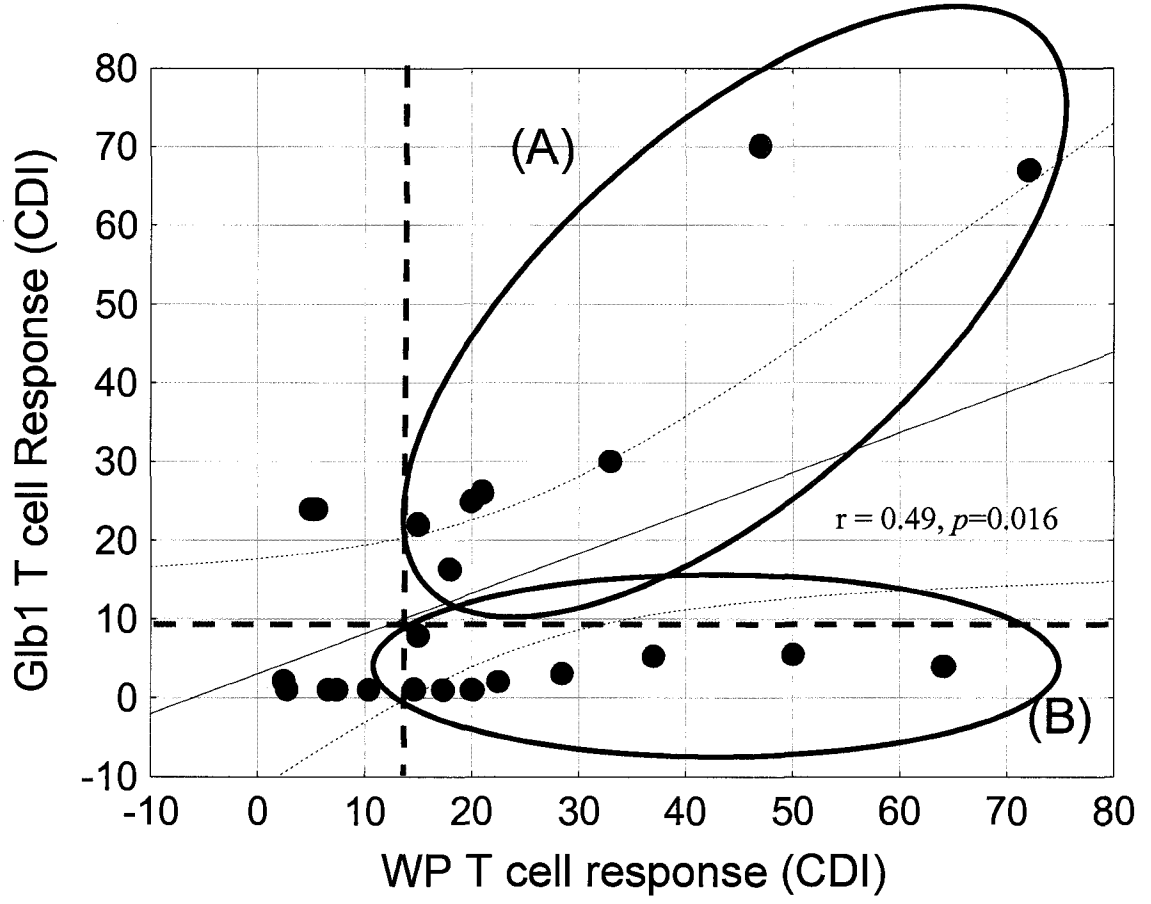
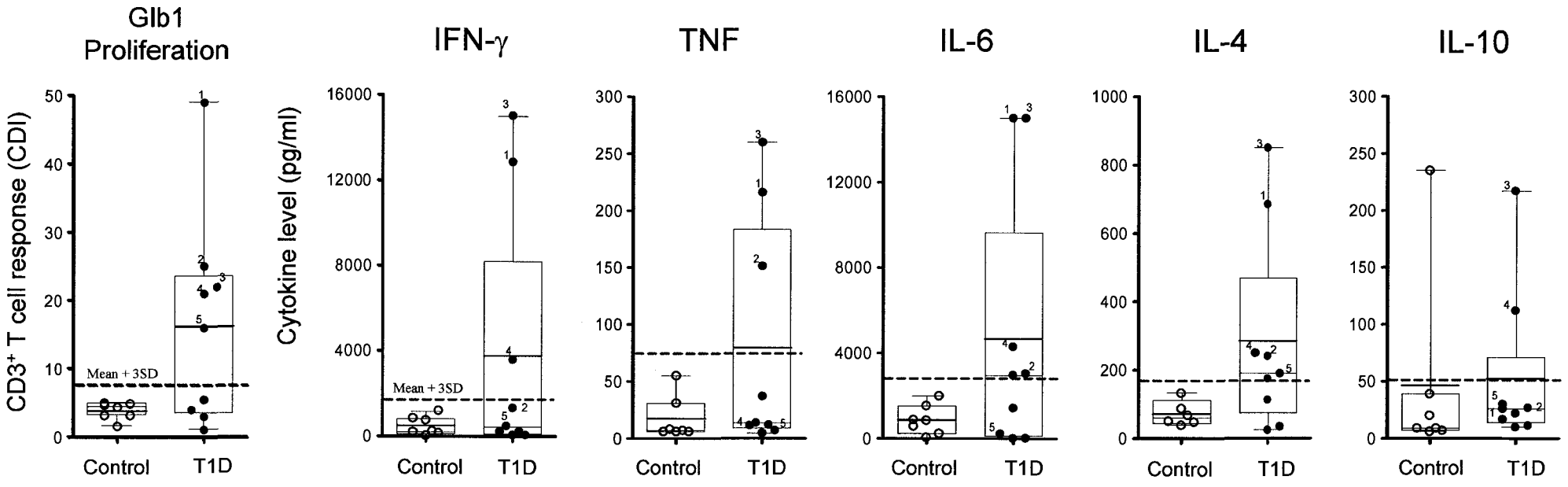

80
70
60
50
40
30
20
10
0
-10
-10
0
10
20
30
40
50
60
70
80
Glb1 T cell Response (CDI)
WP T cell response (CDI)
(A)
(B)
r = 0.49, p=0.016

Figure 6.4 Cytokine concentrations in supernatants from Glb1-stimulated PBMC from T1D patients and controls

Cytokine profiles of supernatants from PBMCs cultured with Glb1 were analyzed on day 8 using Th1/Th2 cytometric bead arrays. Box and Whisker plots with individual values for Control and T1D subjects are shown. Each plot shows the mean (solid line), median (red line), distribution, and range. Some of Glb1-stimulated PBMC of T1D patients showed a Th1 type response, with a high level of IFN-γ and IL-6. The cutoff value (dashed line) labeled on each graph represents the control mean + 3 SD. Five individuals who had a CDI higher than the cutoff value in the proliferation assay are labeled with the numbers (1-5) to more easily follow for their cytokine pattern.


GIb1
Proliferation
IFN-γ
TNF
IL-6
IL-4
IL-10
CD3+ T cell response (CDI)
Cytokine level (pg/ml)
Mean + 3SD
Control
T1D

Glb1 antibody levels increased in a subset of T1D patients

Antibody levels were evaluated in serum or plasma of T1D patients and controls. Of 27 patients with T1D, 7 (26%) had a positive antibody level, using the mean + 2 SD of arbitrary units ≥ 41 in the control group as the cutoff for a positive response. Glb1 antibody levels reported as arbitrary units were significantly different between patients (n=27, arbitrary units=33.4 ± 17.4, mean ± SD) and controls (n=15, arbitrary units=19.5 ± 17.4); median (range) 34 (8 - 78) for the patients and 19 (2 - 36) for the controls (Figure 6.5, p=0.01, Mann-Whitney U-test).

Coincidence of T cell and antibody responses to Glb1 in a subset of T1D patients

There was a positive correlation between T cell response to Glb1 and antibody reactivity in sera of patients (Pearson's r=0.64, p=0.0032). Five out of 6 T1D patients, who displayed a positive proliferative response to Glb1 also had a positive Glb1 antibody response. Only one individual showed positive responses in the T cell assay but not in the antibody ELISA (Figure 6.6).

Table 6.1 Within assay precision for Glb1 assay

Within assay precision for Glb1 assay calculated from 6 samples (one from a patient with high Glb1 antibody level and the others from 5 control individuals with different values of Glb1 antibody) with 10 replicates in one plate.

Samples	Number of replicates	Mean (Arbitrary Units)	Standard Deviation	CV %
1	10	1.1	0.2	12.6
2	10	6.1	0.6	10.0
3	10	10.9	0.3	2.8
4	10	10.6	0.3	2.9
5	10	14.8	0.4	2.4
6	10	92	1.0	1.1

Figure 6.5 Serum Glb1 antibody level in patients and controls determined using ELISA

The mean + 2 SD (>41 arbitrary units) was used as a cutoff to define a positive response. We found a positive antibody response to Glb1 in 7 T1D patients (26%; $p<0.01$ vs. controls, Mann-Whitney U-test).

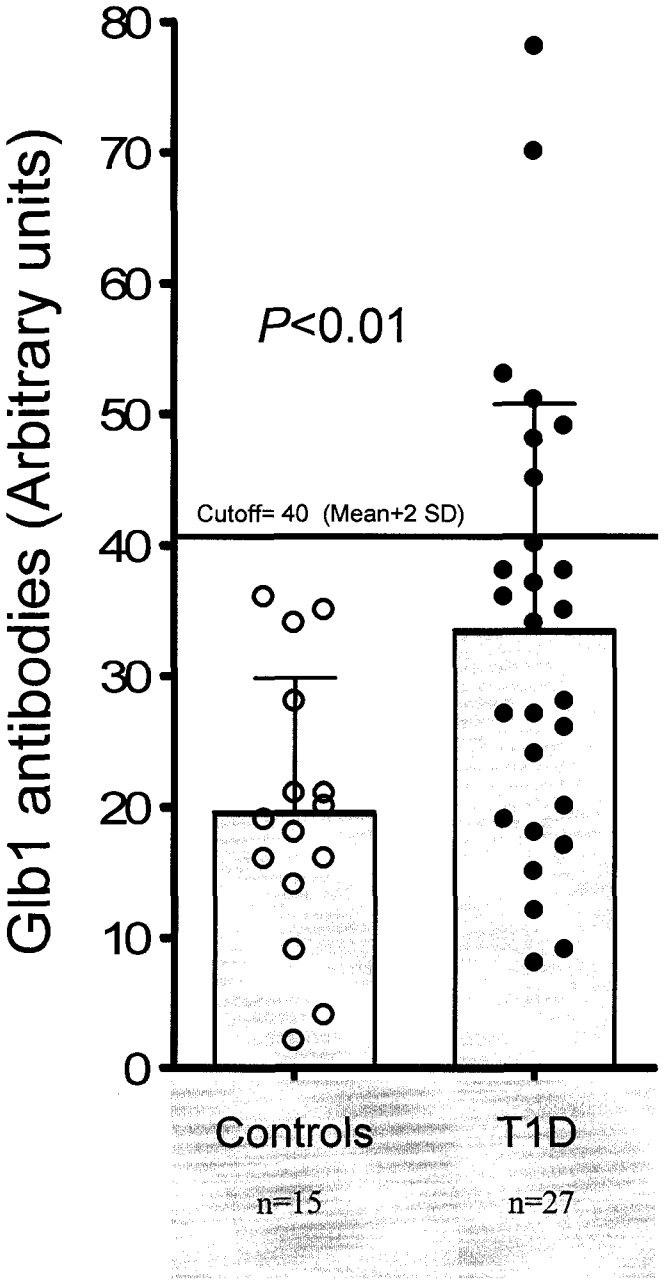
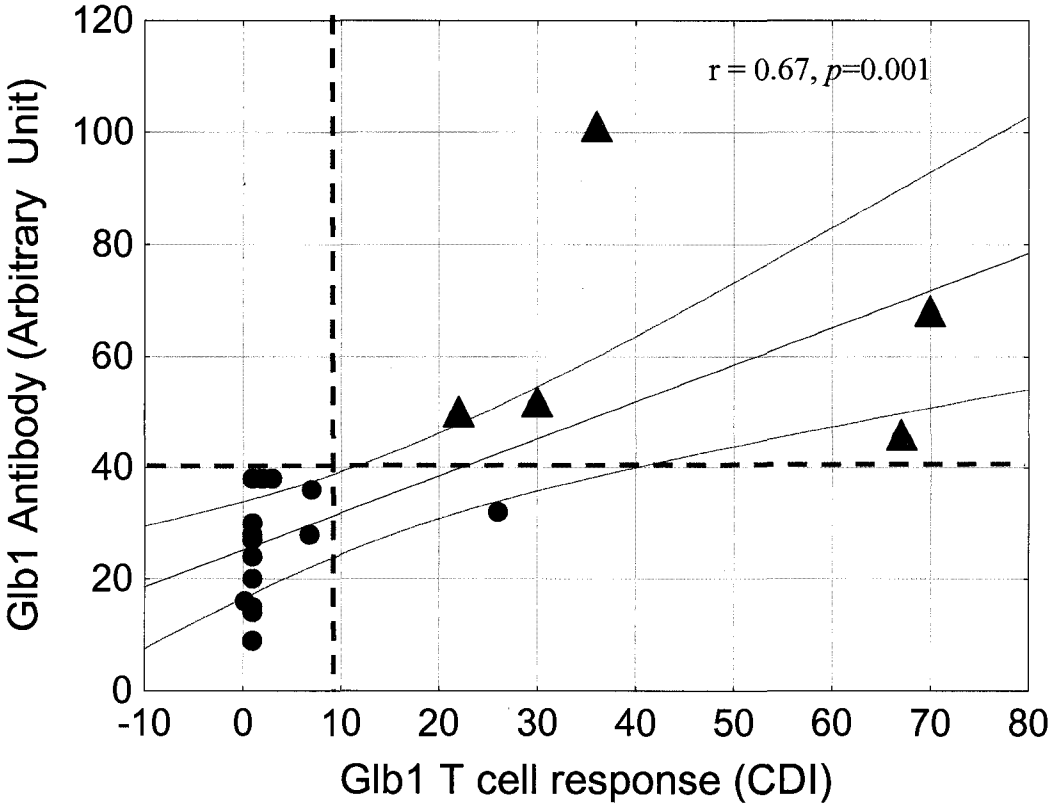

Glb1 antibodies (Arbitrary units)
80
70
60
50
40
30
20
10
0
P<0.01
Cutoff= 40 (Mean+2 SD)
Controls
n=15
T1D
n=27

Figure 6.6 Coincidence of T cell and antibody response to Glb1 in individual T1D patients

Five out of 18 T1D patients (red triangle) displayed both T cell and antibody reactivity against Glb1. One individual with high T cell reactivity displayed antibody levels lower than the cutoff value. Dashed lines show the cutoff value for Glb1 T cell response and antibody responses.


r = 0.67, p=0.001
Glb1 Antibody (Arbitrary Unit)
Glb1 T cell response (CDI)
120
100
80
60
40
20
0
-10
0
10
20
30
40
50
60
70
80

Group 2 Children with T1D islet autoantibodies

T1D is one of the most common chronic diseases in children. The Diabetes Auto Immunity Study in the Young (DAISY) is an observational study at the University of Colorado Health Sciences Center, Denver, USA. This study follows children at increased risk of developing type 1 diabetes, defined as children with a diabetic relative (sibling or parents) or children at an increased genetic risk. The purpose of DAISY is to determine whether there are environmental factors involved in triggering T1D. The main environmental factors being studied are viruses, diet, immunization and social stress. We were approached by colleagues in Denver to evaluate Glb1 antibody reactivity in serum from children in this study. We examined 88 serum samples, from 35 children who developed Islet Autoimmunity (IA) defined as those who were positive for at least two autoantibodies (Insulin, GAD65, IA-2) and being positive at last follow-up (n=16) or developing type 1 diabetes (n=19). In parallel, as a control group, we also examined sera from 63 children at high genetic risk from the DAISY study who have not developed autoimmunity during 4.3 years of follow up.

Results

The IA cases who had positive islet autoantibody results at the time of sampling displayed mean Glb1 antibody levels similar to high-risk controls (mean ± SD; 29.4 ± 39.7 vs. 20 ± 20, p=0.7; Mann-Whitney U-test) (Figure 6.7). To evaluate whether Glb1 antibodies are a risk factor for IA, logistic regression analysis on untransformed Glb1 antibody values was performed by the Denver group. With this analysis Glb1 antibodies were not significantly associated with IA (OR 1.02, CI: 0.997-1.038, p=0.09), suggesting that Glb1

antibodies were not a covariate of IA. However, when they dichotomized untransformed Glb1 antibody values by using the 95th percentile (cutoff=70.0), seven children had a positive value for Glb1 antibody and logistic regression in this situation was marginally associated with IA (OR: 6.18, CI: 0.95-40.35, p=0.057) after adjusting for age, DR4 status, cereal introduction and having a first degree relative with T1D.

To understand further whether Glb1 could be an early predictor of future IA risk, we compared the Glb1 level in the earliest samples (prior to the first positive visit) versus controls (Figure 6.7, p=0.76, Mann-Whitney U-test). This result indicated that elevated Glb1 would not be an early predictor of future IA risk in high risk children. However, there are several caveats which will be discussed in more detail in the discussion section. We also evaluated the variation of Glb1 antibody in each individual at the negative visit and another at a later time point when they became positive for islet antibodies (Figure 6.8). The pattern suggested a relationship between Glb1 antibody and development of islet autoimmunity in a subset of patients.

Figure 6.7 Serum Glb1 antibodies among children with islet autoimmunity (DAISY Study)

The mean Glb1 antibody level at positive and negative visit for IA was not significantly higher compared with controls (p=0.70 and p=0.80 respectively, Mann-Whitney U-test). In both controls and cases, a subset of individuals showed high Glb1 antibody levels. Note that there appear to be two distinct populations in controls: Population one contains values between zero and 22 and population two contains values between 25 to 90 arbitrary units.

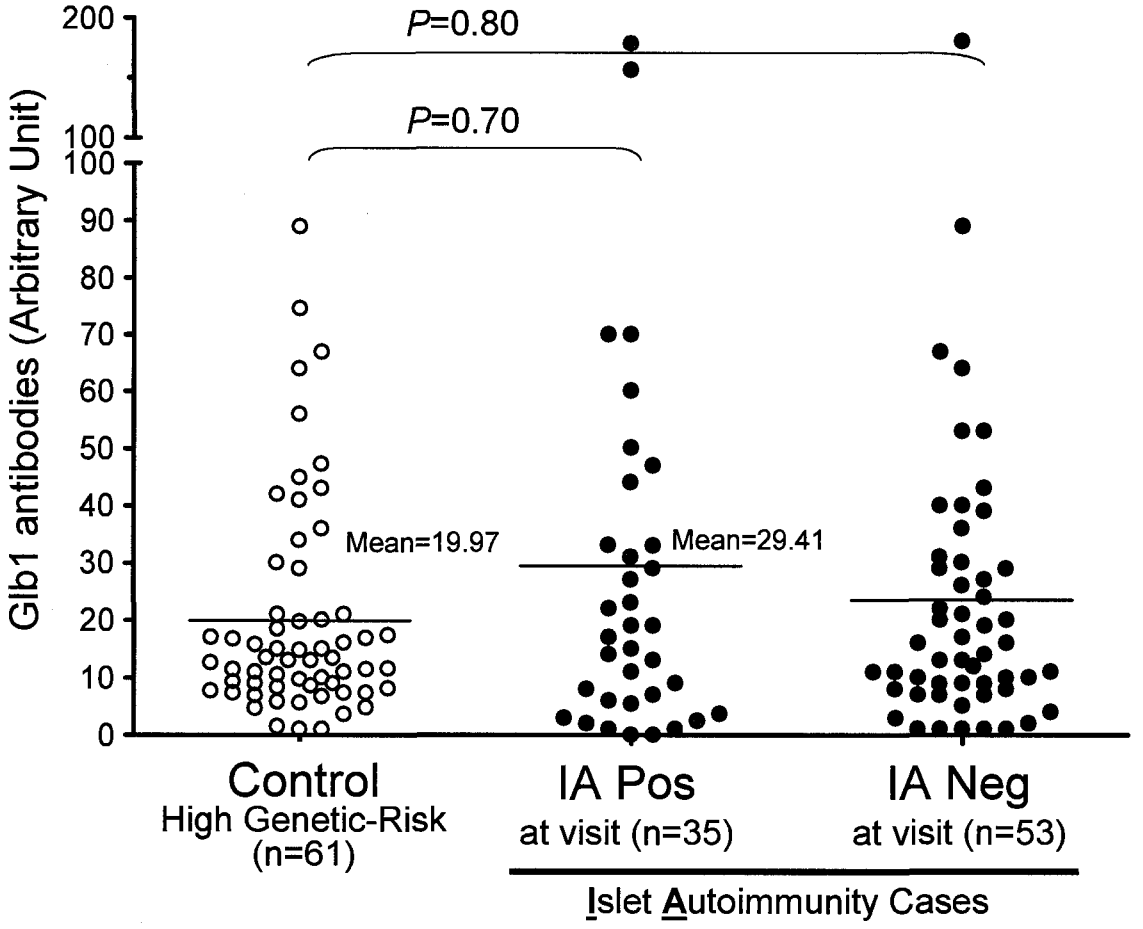
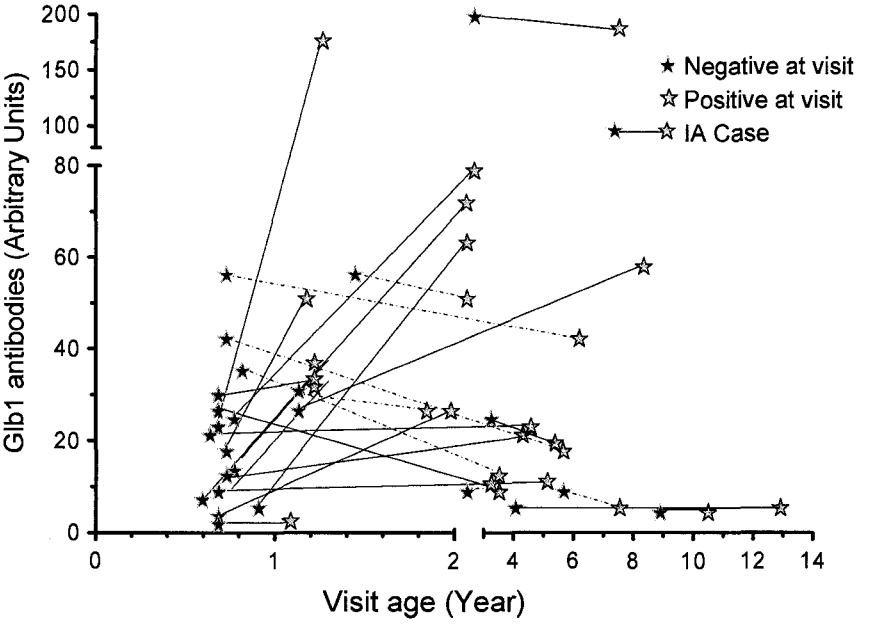

200
100
100
90
80
70
60
50
40
30
20
10
0
P=0.80
P=0.70
Glb1 antibodies (Arbitrary Unit)
Mean=19.97
Mean=29.41
Control
High Genetic-Risk
(n=61)
IA Pos
at visit (n=35)
IA Neg
at visit (n=53)
Islet Autoimmunity Cases

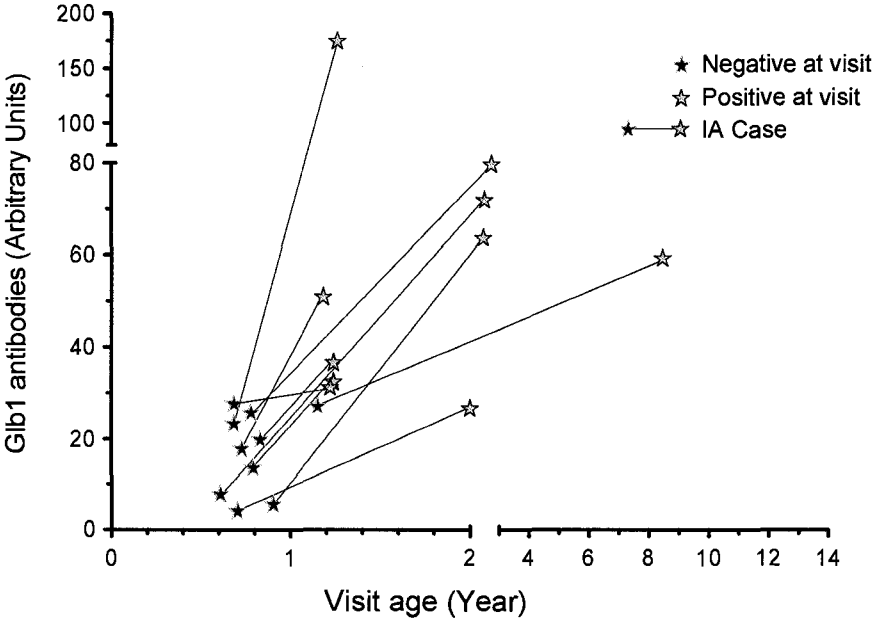
Figure 6.8 Glb1 antibody levels before and after appearance of islet autoimmunity

(A) Different patterns of Glb1 antibody concentration in two samples from individuals with islet autoimmunity. **(B)** group of individuals with strong increase of Glb1 antibody in the second sample when they became positive for islet autoantibodies **(C)** group of individuals with high or intermediate Glb1 antibody level in the first sample with some decrease in the second sample when they became positive for islet autoimmunity (dashed line), and a group of cases who had low and/or unchanged Glb1 antibody levels (solid line).

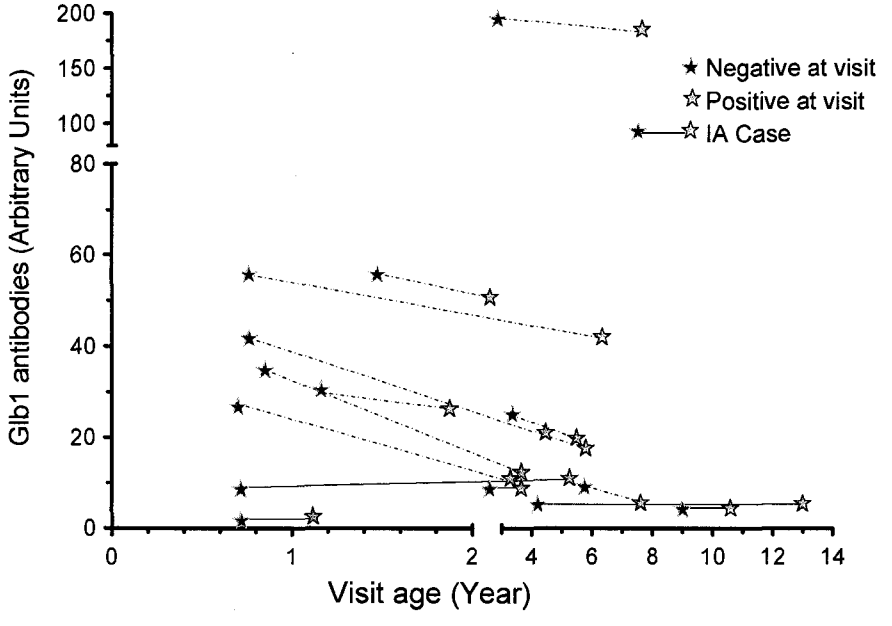
(A)


Glb1 antibodies (Arbitrary Units)
Visit age (Year)
Negative at visit
Positive at visit
IA Case

(B)


Glb1 antibodies (Arbitrary Units)
Visit age (Year)
Negative at visit
Positive at visit
IA Case

(C)


Glb1 antibodies (Arbitrary Units)
Visit age (Year)
Negative at visit
Positive at visit
IA Case

Group 3 Children with coeliac disease autoimmunity (tTG autoantibodies)

Coeliac disease is the most common lifelong chronic disease [125]. Approximately 1-6.2% of patients with T1D have CD [127,131,173]. CD and T1D are both immune-mediated inflammatory disorders involving IFN-γ dependent immune responses. Both diseases share certain genetic risk linked to the HLA-DQ2 and HLA-DR3 genes. There is additional evidence of a link between wheat and coeliac-like reactions in patients with T1D. For example 20-30% of T1D patients have tTG antibodies in their serum which are diagnostic for CD [190]. Furthermore, 20% of children with T1D developed coeliac-like immune reactivity to gliadin that was instilled rectally, suggesting some T1D patients have an abnormal mucosal immune response to wheat [191].

Recently we reported a highly wheat sensitive patient, who had both T1D and coeliac disease (Chapter 5) [143]. This patient showed a strong immune reactivity to Glb1. Understanding whether immune reactivity to Glb1 is associated with T1D or CD or both diseases will be important. Therefore, we analyzed Glb1 antibodies in young tTG^+ children.

Serum samples from the CEDAR study were provided by Dr. Marian Rewers and Dr Jill Norris at the University of Colorado, School of Medicine, Denver, USA. The CEDAR study is evaluating the association between coeliac disease, autoimmunity and environmental factors. The participants in this study were chosen from the DAISY study and are volunteers who have become positive for IgA antibodies to the major coeliac autoantigen, tTG. We evaluated serial samples from 50 children who were either positive for the tTG autoantibodies at two or more consecutive visits or positive for the tTG and /or coeliac-related endomysial autoantibodies (EMA) at least once, followed by a positive small

intestinal biopsy, a total of 217 samples. Also, 50 tTG negative high genetic risk children matched for age at first tTG positive visit, HLA-DR3, DQ2, sex, ethnicity and family history were analyzed as a high-risk control group.

Results

Glb1 antibody level is higher at the time of seroconversion to tTG IgA

All samples from cases who became positive for tTG had higher Glb1 antibody concentration compared with controls (Arbitrary unit mean ± SD, 24.2 ± 20.5 vs. 16.5 ± 14.6, p=0.002, Mann-Whitney U-test) (Figure 6.8). Interestingly, this significant difference was also observed in tTG^+ cases on their last tTG^- visit and at first positive visit compared with controls (23.23 ± 18.0, p=0.005; 23.3 ± 21.3, p=0.007 respectively) (Figure 6.10, Table 6.2). Advanced logistic regression analyses performed by our colleagues in Denver also showed that after adjusting for age, breast feeding duration and timing of gluten exposure, cases had significantly higher Glb1 antibody than controls (OR 1.8, 95% CI 1.1-2.9). Glb1 antibody levels were associated with younger age and tTG-positivity and marginally associated with longer breast-feeding and later age at gluten introduction. Glb1 antibody levels were higher in HLA-DR3, DQ2 negative vs. positive cases. Glb1 antibody levels were associated with first exposure to wheat in cases but not in the controls [192].

IgG Glb1 antibodies are increased before IgA-tTG in subjects with CD

We also evaluated the distribution of Glb1 antibody level and IgA-tTG antibody in all samples taken at different ages (Figure 6.11). Glb1 level peaked at age 2-3 years and then declined in most cases. By contrast, tTG levels peaked after 3 years. The Denver analysis

confirmed that Glb1 antibodies appeared before tTG antibodies and coeliac disease but Glb1 was not associated with tTG-IgA level over time.

We also evaluated the variation of Glb1 antibodies and tTG-IgA in each individual in different ages until they became positive for tTG-IgA (Figure 6.12). The pattern suggested Glb1 antibodies frequently occurred at an earlier age than coeliac disease autoimmunity. In most cases Glb1 antibodies peaked and declined before tTG-IgA became positive in young high-risk children.

Figure 6.9 Serum Glb1 antibodies in cases and controls analyzed using ELISA (CEDAR study)

217 serial samples from 50 IgA-tTG$^+$ cases at different ages and 50 high-genetic risk controls were evaluated for Glb1 antibody level (Denver, CEDAR Study). The mean Glb1 antibody value of all samples from cases[1] was significantly higher compared with controls (24.16 ± 20.46 *vs.* 16.5 ± 14.6 (SD), p=0.002, Mann-Whitney U-test).

[1]Samples from cases = 217 samples consisted of two or more samples from 50 children who were either positive for tTG autoantibodies or became positive at a consecutive visit.

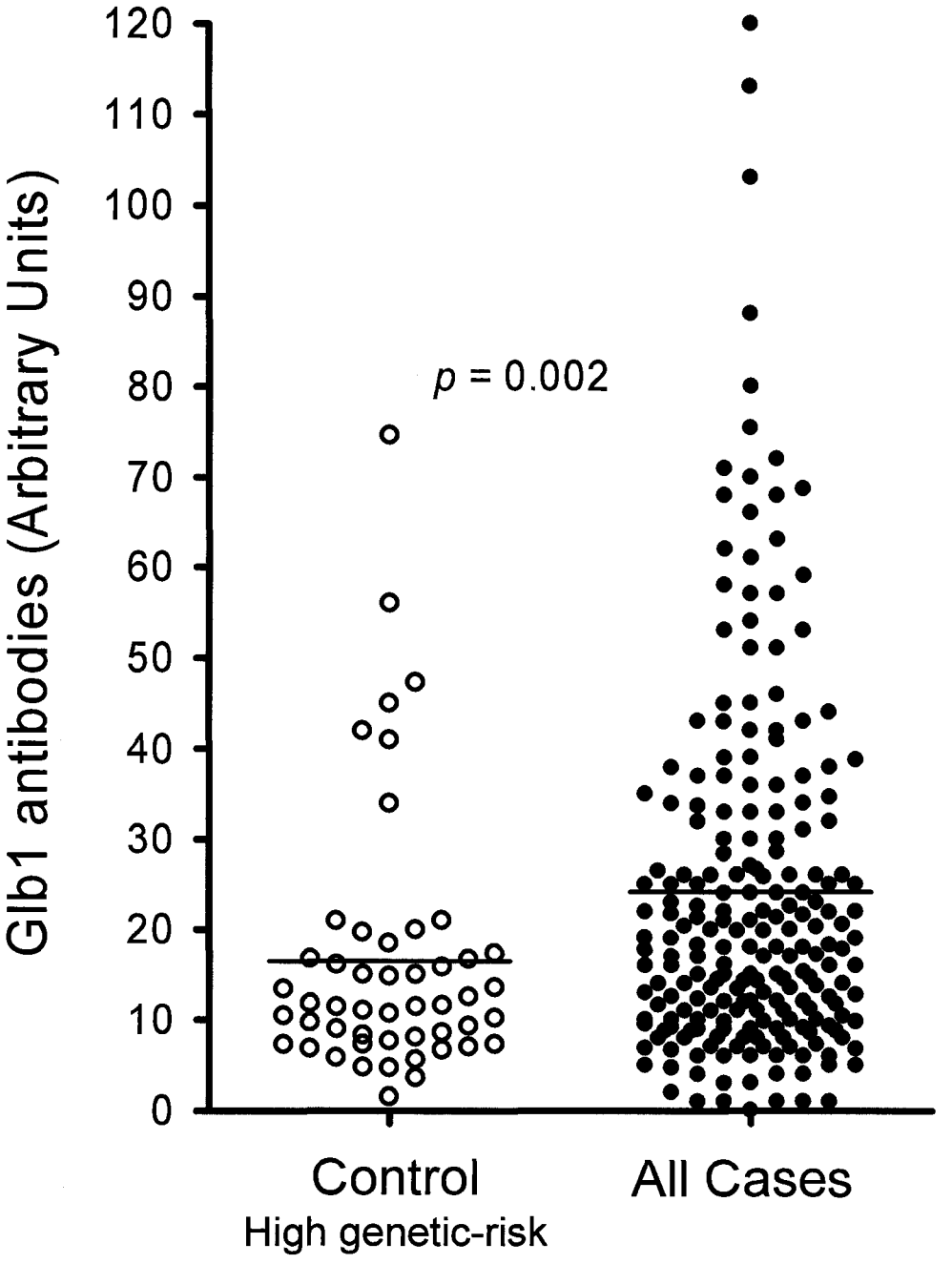

Glb1 antibodies (Arbitrary Units)
120
110
100
90
80
70
60
50
40
30
20
10
0
p = 0.002
Control
All Cases
High genetic-risk

Table 6.2. Glb1 antibody level and age of children at risk of developing Coeliac Disease (CEDAR study)

Comparing age and Glb1 antibody level between high genetic-risk control and coeliac cases in all negative samples for tTG, last negative visit and first positive visit for tTG. The mean Glb1 antibody value of all negative samples from cases was significantly higher compared with controls (p=0.004). The mean of samples, collected at the last negative visit and first positive visit also showed a significant difference compared with the control group (p=0.005 and p=0.007 respectively, Mann-Whitney U-test)

	Controls (n=50)				Cases (n=50)								
				P value for difference	*All negative Visit Samples (n=117)*			*Last Negative Visit Samples (n=50)*			*First Positive Visit Samples (n=50)*		
	Mean	**SD**	**Range**		**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Glb1 antibodies (Arbitrary units)	16.5	14.6	1.5-74	0.004 *vs.* All negative visit 0.005 *vs.* Last negative visit 0.007 *vs.* First Positive visit	24.9	21.2	0.1-113	23.2	18.0	6.0-88	23.3	21.3	4.7-120
Age at sample	4.9	1.9	1.9-10.7	0.0001 *vs.* All negative visit 0.0003*vs.* Last negative visit 0.9 *vs.* First Positive visit	2.2	1.6	0.4-7.4	3.7	2.0	0.7-9.9	4.9	1.8	2.0-11

Figure 6.10 Serum Glb1 antibodies in cases based on visit type (CEDAR study)

Comparing Glb1 antibody level between high genetic-risk control and Coeliac cases in all negative samples for tTG, last negative visit (green) and first positive (red) visit for tTG. The mean Glb1 antibody value of all negative samples from cases was significantly higher compared with controls (p=0.004, Mann-Whitney U-test). The mean of samples, collected at the last negative visit (green) and first positive visit (red) also showed a significant difference compared with the control group (p=0.005 and p=0.007 respectively, Mann-Whitney U-test). Note that there appear to be two distinct populations in controls.

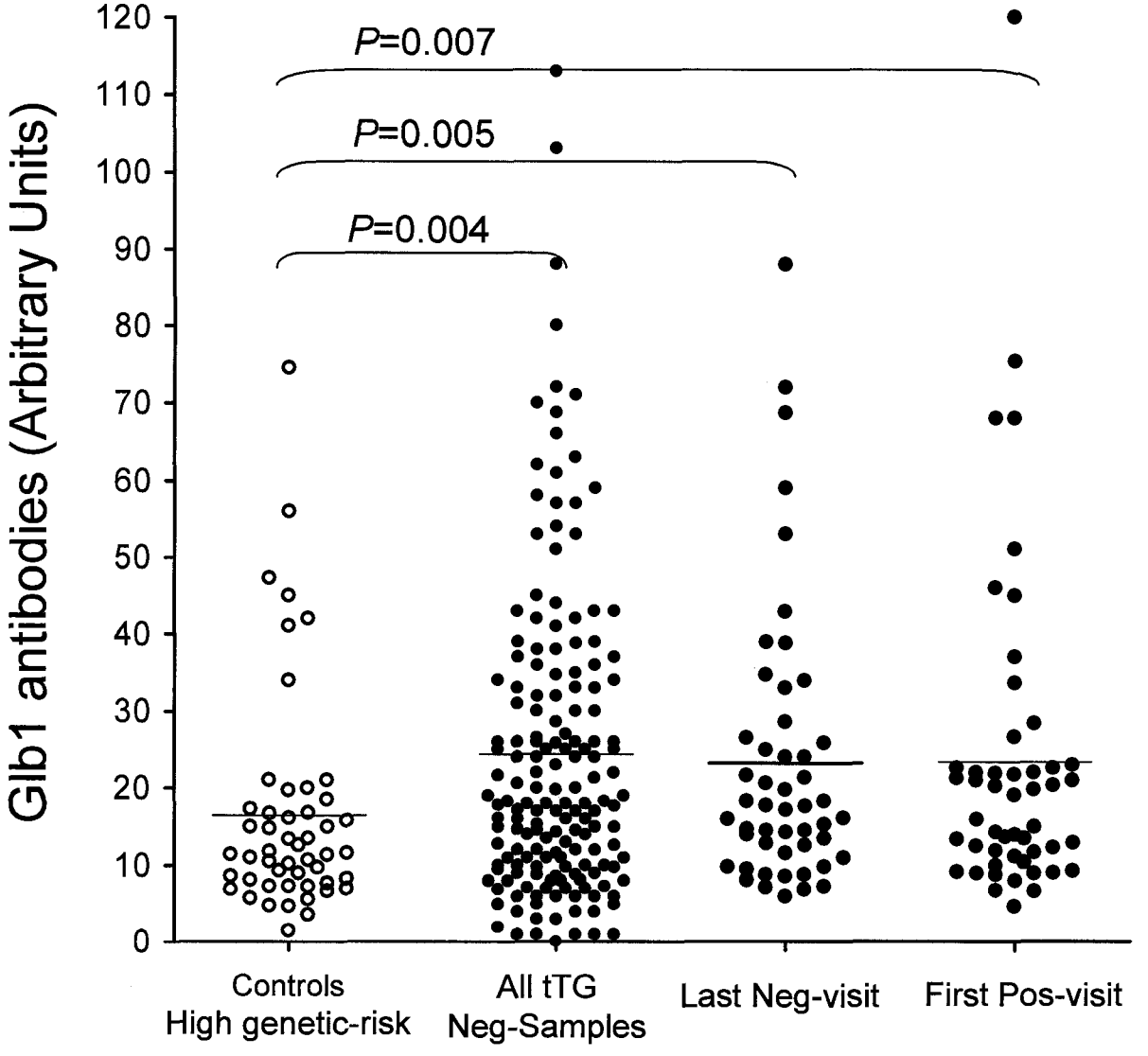
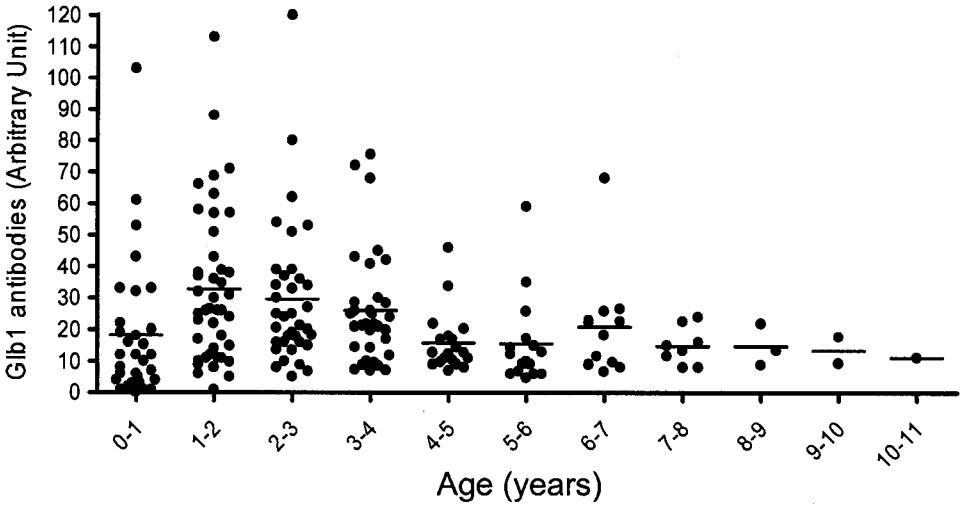

Glb1 antibodies (Arbitrary Units)
120
110
100
90
80
70
60
50
40
30
20
10
0
P=0.007
P=0.005
P=0.004
Controls
High genetic-risk
All tTG
Neg-Samples
Last Neg-visit
First Pos-visit

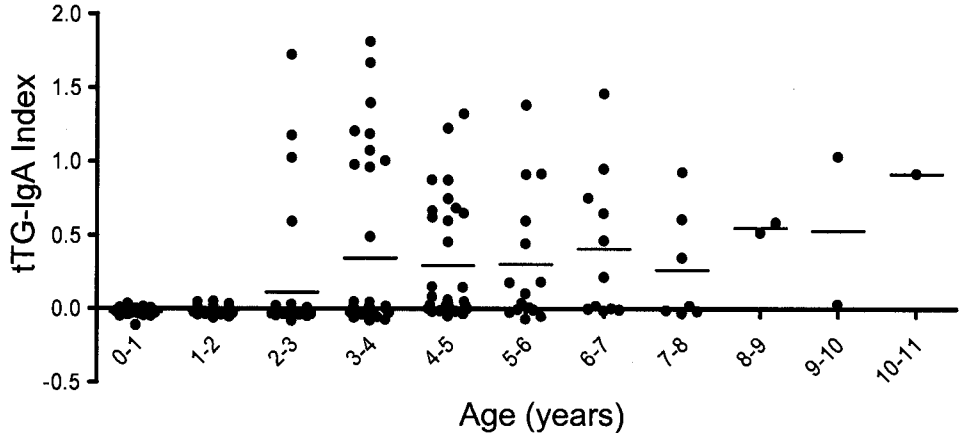
Figure 6.11 Comparison of Glb1 antibody level and IgA-tTG antibodies in coeliac disease autoimmunity (CDA) cases

(A) Distribution of Glb1 antibody level in 217 samples taken at different ages in 50 CDA cases from Denver. **(B)** Distribution of IgA-tTG antibody level in the same samples in (A). Glb1 antibody peak occurred at about age 1-3 years and then declined. By contrast, IgA-tTG antibodies appeared later and peaked at approximately 3-5 years of age. **(C)** IgG-Glb1 antibodies (green) appear before IgA tissue transglutaminase antibodies (yellow) in young high-risk children.

(A)


Glb1 antibodies (Arbitrary Unit)
120
110
100
90
80
70
60
50
40
30
20
10
0
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
Age (years)

(B)


tTG-IgA Index
2.0
1.5
1.0
0.5
0.0
-0.5
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
Age (years)

(C)

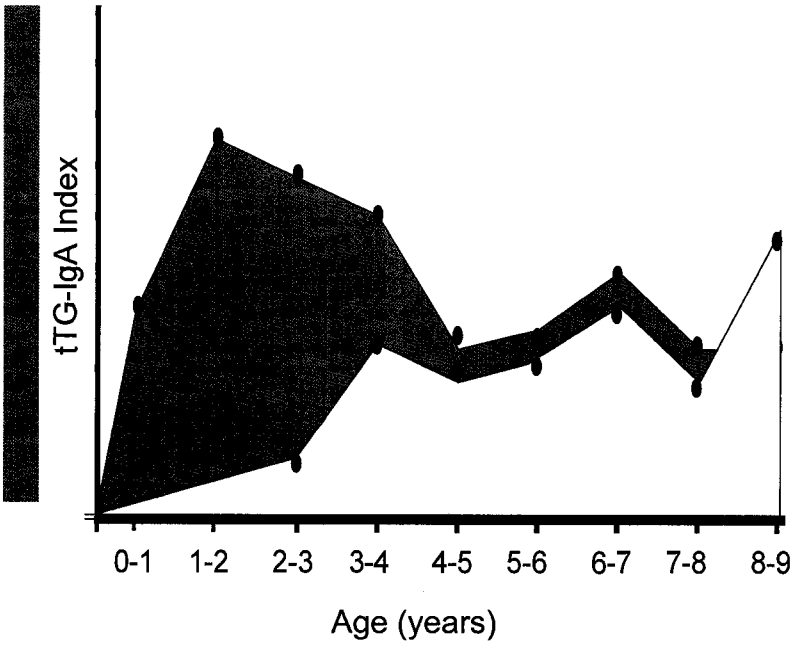
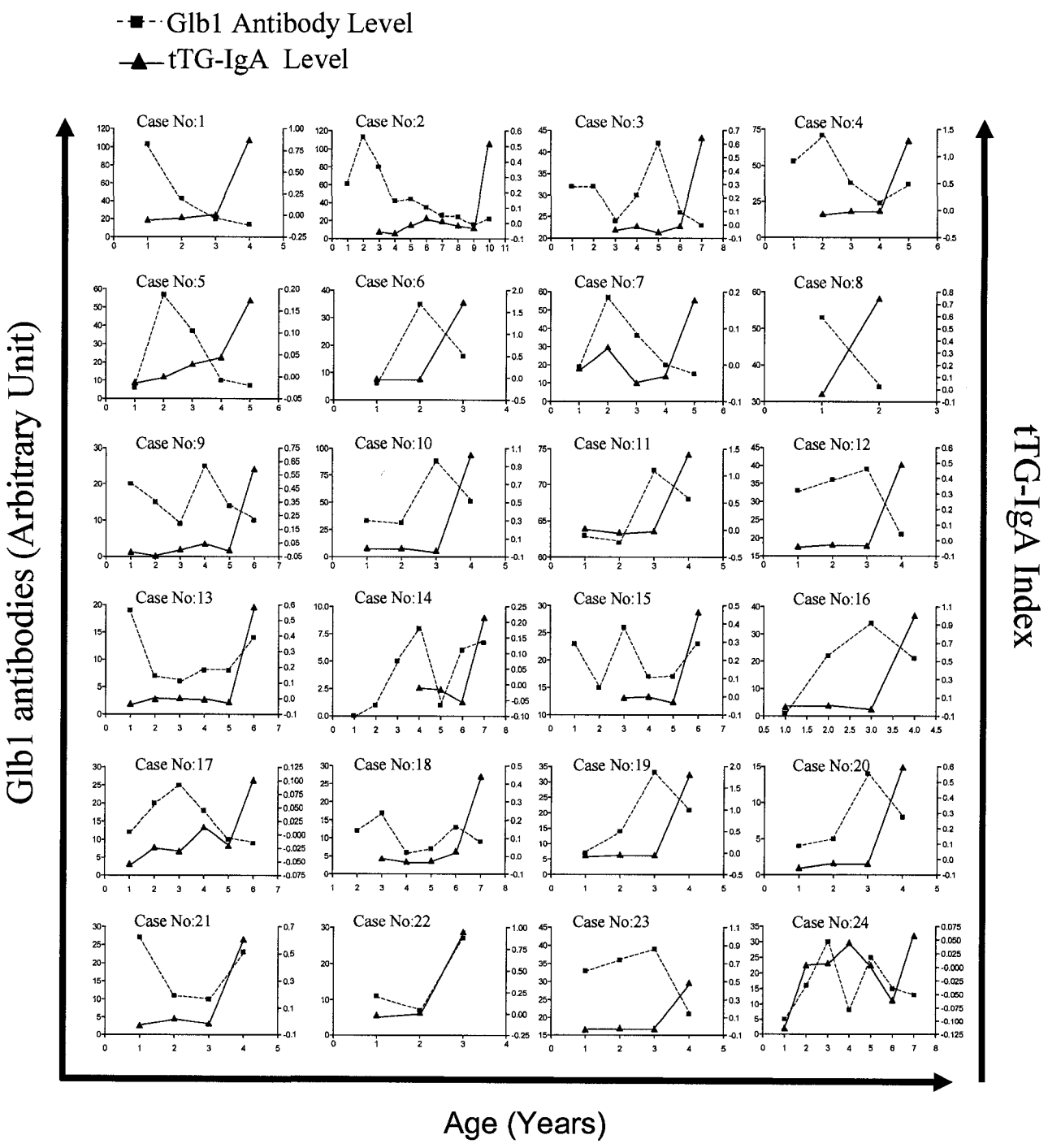

tTG-IgA Index
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
Age (years)

Figure 6. 12 Development of Glb1 antibodies frequently precedes or coincides with tTG-IgA seroconversion

Variation of Glb1-IgG antibody (-◆-) and tTG-IgA level (—▲—) at different ages was compared in 24 Coeliac Disease Autoantibody (CDA) cases from Denver. In several cases a peak with high level of Glb1 is present years before the cases became positive for tTG. There was frequently an inverse relation between tTG and Glb1, such that the level of Glb1antibody was high, tTG -IgA was low (e.g. cases No:1-7 and 10-12). Even in cases when Glb1 antibody level was low, a pattern was present in which Glb1antibodies peaked before tTG-IgA (e.g. cases No: 13-15 and 20-21). tTG-IgA level is expressed as an index which is defined as: (unknown sample cpm − negative control cpm)/(positive control cpm − negative control cpm)[1].

[1] Bao, F., L. Yu, S. Babu, T. Wang, E. J. Hoffenberg, M. Rewers, and G. S. Eisenbarth. 1999. One third of HLA DQ2 homozygous patients with type 1 diabetes express celiac disease-associated transglutaminase autoantibodies. *J.Autoimmun.* 13:143-148.


Glb1 Antibody Level
tTG-IgA Level
Case No:1
Case No:2
Case No:3
Case No:4
Case No:5
Case No:6
Case No:7
Case No:8
Case No:9
Case No:10
Case No:11
Case No:12
Case No:13
Case No:14
Case No:15
Case No:16
Case No:17
Case No:18
Case No:19
Case No:20
Case No:21
Case No:22
Case No:23
Case No:24
Glb1 antibodies (Arbitrary Unit)
tTG-IgA Index
Age (Years)

Summary and Discussion

Study Group 1 Young adult T1D patients

We evaluated immune reactivity against Glb1 in PBMC of young T1D patients. Glb1 was recently identified as the first diabetes-related wheat protein in diabetes-prone rats [86]. T cell reactivity to wheat proteins described in Chapter 3 of this thesis indicated that more than 45% of young adult T1D patients had abnormal T cell immune responses to WP. Here we show in a small subset of T1D patients, around 40% of them have abnormal T cell reactivity to Glb1 (Figure 6.1). Interestingly, we found a positive correlation between WP and Glb1 T cell responses in some patients (Figure 6.3). Glb1 is a minor contaminant of WP, which is an extract of wheat gluten. Therefore, we cannot necessarily attribute the immune reactivity to WP in cases who also showed immune reactivity to Glb1 to a small contamination of WP with Glb1. In addition, nine individuals showed T cell reactivity to WP but not to Glb1 (Figure 6.3), confirming that Glb1 is not the only immunogenic protein in the complex of wheat proteins.

The HLA class II genes are the major genetic determinant of T1D and our data demonstrate that monoclonal anti-DR antibodies blocked the T cell proliferative response to Glb1 in T1D patients (Figure 6.1 B) indicating that these T cell responses were associated with HLA-DR.

There are several reports confirming the contribution of antibodies to the pathogenesis of some autoimmune diseases, but to date there is no strong evidence to show a direct contribution of antibodies to β-cell damage [115]. Previous studies from our laboratory showed that antibody reactivity to Glb1 was correlated with islet inflammation and damage

in BBdp rats [86]. Unpublished preliminary data from my ongoing study indicated that Glb1 antibodies generated in rabbit are able to bind to a 62 KDa protein from human islets.

In the present study, we evaluated the Glb1 antibody level in serum of T1D patients. As many as 26% of T1D patients had abnormally high Glb1 antibody levels. In these patients we observed both increased T cell and antibody response to Glb1 (Figure 6.6). Five out of 6 T1D patients, who had a positive T cell response to Glb1 also showed a positive level of antibody. Some Th1 diseases such as rheumatoid arthritis, multiple sclerosis and diabetes could be mixed Th1/Th2 conditions [193,194]. Interestingly, in the current study we showed strong T cell reactivity to Glb1 and cytokine analysis in a subset of T1D patients revealed that some individuals with high Glb1 T cell reactivity also produced high levels of IFN-γ and IL-6 in response to Glb1 (Figure 6.4). Notably, the Th2 cytokine, IL-4, was also higher than the cutoff value (mean + 3 SD of control subjects) in response to Glb1 indicating the presence of a Th2 response in parallel with the high level of the Th1 cytokine, IFN-γ (10-20 fold higher than IL-4 level). This might explain in part the presence of a strong IgG reactivity to Glb1 in some of our patients. Th1 cells are relatively poor initiators of antibody responses but they participate in isotype switching by producing IFN-γ, which drives B cells to secrete Th1-associated IgG2α and IgG3 [195]. The biological functions of IgG antibodies depend on their subclass. IgG1 and IgG3 subclasses have been shown to be mediators for antibody dependent cell cytotoxicity (ADCC). They are also able to bind complement factor C1 with high efficiency. In contrast IgG2 and IgG4 are weak activators of the complement system, and it has been suggested that these subclasses of antibodies prevent ADCC by masking target cells [196]. It has also been suggested that in autoimmune diseases high-avidity autoantibodies may play a role in the pathogenesis of disease [197]. Further study is required to

evaluate the subtype of antibodies in individuals with high Glb1 antibody levels to determine the avidity of these antibodies in samples, collected before and after development of islet autoimmunity.

Study Group 2 Children with islet autoimmunity (DAISY)

To evaluate whether the immune reactivity to Glb1 was present in a younger high risk population, we analyzed Glb1 antibody levels in archived sera from children at increased genetic risk for T1D from the DAISY study. Results showed that the mean Glb1 antibody level was higher in children who became positive for islet autoantibodies compared with high risk controls, but this difference was not statistically significant (Figure 6.7). The simplest interpretation of this finding is that Glb1 antibodies do not differentiate children with islet autoimmunity from controls coming from a high risk population. However, several points need to be considered. There are two distinct populations in the control group based on Glb1 antibody level (Figure 6.7). The control group was actually chosen from high genetic risk children, who did not develop autoimmunity during 4.3 years of follow-up. Because the mean age at onset of T1D is 13 years [8], we do not know whether the high Glb1 individuals will go on to develop T1D autoimmunity or overt diabetes at a later age. Another point is that the control sample group was age matched with samples collected at the time when islet autoimmunity was first detected. However these samples were not matched with samples collected at earlier ages (Table 6.2). Therefore, it will be important to follow up control individuals to monitor the development of IA at a later age. Alternatively,

it will also be important to use non-high risk controls to ascertain whether Glb1 antibody concentration could be a predictor of diabetes risk in some individuals.

Evaluation of samples from IA cases, showed three different patterns for Glb1 antibody levels. Two samples were collected from each individual, one at the negative visit and another at a later time point when they became positive for islet antibodies. Figure 6.8 A clearly shows a strong increase in Glb1 antibody level between the two sample points in some individuals but not in others (Figure 6.8). These patterns of Glb1 antibody changes with time suggest a relationship between Glb1 antibody and development of islet autoimmunity in a subset of individuals. To clarify this relationship we need to expand this study and analyze several prospective samples from cases and controls. These results could help answer the question whether Glb1 antibody level can be used as an early marker of diabetes risk before appearance of islet autoantibodies.

Several reports indicate increased gut permeability in an animal model of diabetes and in human T1D patients [97,99,100,144,145]. Increases in zonulin, a protein secreted by gut epithelial cells, could increase intestinal permeability by disassembling the intercellular tight junctions [198]. There is one report indicating that increased gut permeability is required for development of T1D in BBdp rats [101]. Furthermore, Sapone *et al.* [137] reported that 42% of T1D patients had increased serum zonulin levels and zonulin levels were associated with increased intestinal permeability in a subgroup of T1D patients. Unpublished data from our group and Dr. Norris's group in Denver indicate there were no significant differences in Glb1 antibody and zonulin level between children with and without IA. However, in children with IA, zonulin and Glb1 antibodies were significantly correlated both before and at the first positive samples (respectively, Spearman r=0.60, p=0.0013 and r=0.46, p=0.005). In remarkable contrast, no relation was observed in children without IA (r=0.15, p=0.61). Co-

variation of zonulin and Glb1 antibodies in IA cases but not in IA negative controls suggests that high-risk children who develop diabetes or became positive for islet antibodies may have gut abnormalities related to tight junctions. On the other hand, it is possible that a combination of Glb1 antibody and zonulin level could be a biological marker of diabetes-related gut abnormality.

Study Group 3 Children at risk of developing coeliac disease

The abnormal immune response to Glb1 in the case of a highly wheat sensitive patient described in chapter 5 [143] raised the question whether the response was associated with T1D or CD. In general, globulins including Glb1 are not considered to be coeliac antigens [189]. In addition, Glb1 was discovered by screening a wheat cDNA library with sera from diabetic rats [86,179]. Therefore, we evaluated Glb1 antibody level in cases of young subjects at high risk for T1D who developed coeliac disease autoimmunity. Cases who became positive for tTG showed higher Glb1 antibody levels compared with controls who did not develop CD autoimmunity despite having the same high genetic risk background (Figure 6.9). The presence of a distinct population in the control group including seven individuals with high Glb1 antibody levels (Figure 6.9 and 6.10) will re-open our previous discussion, that some of these control individuals could develop diabetes and/or CD at a later age.

Comparing Glb1 and tTG antibodies at different ages (Figure 6.11 A, B and C and Figure 6.12) indicated that Glb1 antibodies peaked at an earlier age than tTG antibodies. Therefore, an increase in Glb1 antibody before the appearance of coeliac disease autoimmunity in a subset of individuals suggested these antibodies could reveal early

abnormal response to a dietary antigen in those who are asymptomatic but at high risk for T1D who go on to develop CD. This interpretation must be tempered by the small sample size. Our data suggest that the value of Glb1 antibodies as a biomarker of risk for CD or T1D is age related.

Conclusion:

The Gut-Associated Lymphoid Tissue (GALT) is the largest lymphoid organ in the body and is responsible for providing a first line of defense. In addition, it is a highly developed adaptive immune system that regulates the responses to antigens within the gut lumen and those that have crossed the gut barrier. The intestinal immune system is designed to develop oral tolerance to harmless soluble dietary proteins or commensal bacteria [177]. Several factors can influence oral tolerance including, nature and dose of antigen, maturation of immune cells specifically DC as a key APC for priming of naïve T cells [199-201]. Evidence is accumulating in support of a key role for a dysfunctional gut barrier and the presence of a pro-inflammatory condition in the gastrointestinal tract in a variety of human diseases including diabetes, Crohn's disease, coeliac disease, atopic dermatitis, rheumatologic conditions and irritable bowel syndrome [101,145,201,202]. Entry of excessive amounts of dietary antigens via a leaky gut barrier could cause an imbalance in the gut immune system resulting in a breakdown of tolerance and development of one or more of these diseases depending on the presence of risk genes.

Most of the evidence linking the development of diabetes in human to wheat comes from epidemiological studies. Results from this part of my study indicate that a large subset of young adult T1D patients display an abnormal humoral and cellular immune response to

Glb1, a dietary protein. Also, the correlation between Glb1 antibody and zonulin, a marker of gut permeability in a subset of individuals with islet autoimmunity, provides additional evidence that the presence of this dietary antigen in the context of increased gut permeability could induce abnormal immune responses. The abnormal immune responses to Glb1 were also present in children chosen on the basis of high genetic risk for diabetes and who developed CD autoimmunity. It is notable that antibody response to Glb1 was observed before appearance of antibodies to the CD autoantigen, tTG [192]. Therefore, current data suggest the presence of a dysfunctional gut barrier in T1D patients which could permit entry of unusually high amounts of dietary antigens including Glb1 to gut lumen. This excessive amount of antigen could cause an imbalance in the gut immune system and breakdown of tolerance resulting in an abnormal immunity to Glb1. Future study is required to evaluate, whether abnormal immunity to Glb1 may play a role in the pathogenesis of these diseases.

Chapter Seven

Summary and discussion

Summary and general discussion

T1D is a complex, multifactorial autoimmune disease and similar to other autoimmune diseases, it is influenced strongly by genetic factors. Different environmental agents including dietary components have been proposed to promote the autoimmune process 29. There are several reports that link wheat with increased risk of autoimmune diabetes [79-81,149]. Wheat is the most important source of protein in the human diet [76]. In general, wheat and other dietary antigens are harmless in healthy individuals, because of oral tolerance [177], a state of non-responsiveness that originates in the gut immune system. In contrast, wheat can be harmful in some susceptible individuals. Wheat protein is a complex mixture of at least 1300 polypeptides [76], and is responsible for coeliac disease, IgE mediated Baker's asthma and other food allergies [77,78,148]. Wheat is particularly antigenic and immunogenic in human. For instance, gluten proteins in wheat are involved in immune-mediated damage to the gut in CD patients and also in food allergies due to different wheat proteins such as α-, β-, γ-, ω-gliadins and the albumin /globulin fraction [203,204]. In addition, there are several reports that link wheat with increased risk of autoimmune diabetes [79,87,88,149]. However, to date the identity of individual diabetes-related wheat proteins and potential mechanisms involved in the pathogenesis of disease are not known.

The main question in this study was whether there is an abnormal immune response to a mixture of wheat proteins as would be encountered in the daily diet or a purified recombinant wheat storage globulin homologue, Glb1 in T1D patients. In addition to addressing this question, we determined the nature of immune reactivity to wheat proteins and also assessed whether this reactivity was genetically controlled by specific HLA alleles.

To evaluate immune responses to wheat proteins I measured T cell responses using a sensitive CFSE proliferation assay, which we standardized in our laboratory. To date I am only aware of one report that evaluated the T cell immune responses against wheat gluten in T1D patients [85]. In this study, a standard thymidine incorporation assay was used to measure the PBMC response to a specific wheat gluten fraction, Frazer fraction III. The investigators reported low responses in both patients and controls, possibly due to the technique used and the presence of very high concentrations of wheat protein (400 ug/ml of pepsin and trypsin digested wheat gluten), which are in the toxic range [159].

Our CFSE-based assay permitted us to analyze $CD3^+$ T cell proliferation over a period of 8 days in culture (Figure 3.1). This technique has the added benefit of permitting the detection of T cells present at very low frequencies with good reproducibility. Therefore, it was possible to use very low, non-toxic concentrations of wheat protein (6.2 μg/ml; Figure 3.2).

In chapters 3 and 6 of this thesis, we demonstrated high T cell responses to WP and Glb1 in 48% and 40% of young adult T1D patients respectively (Figure 3.3 and 6.1 A). Cytokine analyses of culture supernatants from PBMCs stimulated with WP and Glb1 showed a Th1 type response with high concentrations of IFN-γ and IL-6 (Figure 3.5 and Figure 6.4, patients number 1, 3 and 4). Patients who responded to wheat protein were predominantly HLA-DR (Table 3.4) and immune response to WP and Glb1 was restricted to HLA-DR4 (Figure 3.7 and 6.1 B) and was not associated with HLA-DQ2 (Table 3.4 and Figure 3.7). The only available similar report from Klemetti *et al.* [85] showed increased cell-mediated immune response against wheat gluten in 24% of newly diagnosed T1D patients and 15% of patients with longer duration of disease. Similar to our results,

they did not link gluten-induced T cell responses to HLA-DQB1*02, the major risk allele for coeliac disease or HLA-DQB1*03, a common risk allele in T1D.

In the last several years there are several reports indicating a link between intestinal immunity and T1D [145]. Reports confirm that dietary factors are able to modify diabetes in animal models of autoimmune diabetes and in humans [59,79,87,89,91]. Increased gut permeability is present in prediabetic and diabetic BBdp rats [144,205]. Increased permeability in diabetes-prone individuals could allow abnormal exposure to dietary antigens, which would be expected to disrupt oral tolerance to certain dietary proteins [206]. We have shown that T1D patients also have an abnormal T cell response to other dietary antigens including OVA, gliadin and gliadin 33-mer peptide (Figure 3.3). However, the magnitude of these responses was much lower compared with the response to the WP mixture. In addition, the frequency of individuals who were categorized as responders to these antigens was also lower compared with WP and Glb1 [207] (Chapter 3). This observation strongly suggests that the gut is not functioning normally and raises the possibility of a defect in the gut barrier, an immune imbalance and/or impaired oral tolerance.

Increased gut permeability in human T1D patients has been reported in several studies [97-100]. Zonulin, which regulates intercellular tight junctions, plays a key role in pathogenesis of T1D in BBdp rats [101]. Blocking of the zonulin receptor reduced the T1D incidence by 70% and prevented development of islet cell autoantibodies in these animals. A human study showed that serum zonulin levels were high in 42% of type 1 diabetic patients and this level was associated with increased permeability[137]. In the analyses of the DAISY samples, zonulin levels in children with islet autoimmunity were significantly correlated with Glb1 antibodies [208] (Chapter 6). Importantly, co-variation of Glb1 antibody and zonulin level was absent in IA negative controls. This supports the hypothesis that high-risk children

who became positive for IA or developed diabetes could have increased gut permeability or damage and this defect could in turn permit an excess amount of environmental non-self antigens to stimulate the GALT followed by development of autoimmunity. Additional studies will be necessary to determine whether Glb1 antibody and zonulin level together represent a new biological marker of gut abnormality.

There is no strong evidence to support a direct contribution of antibodies to beta cell damage [115]. Autoantibodies such as Insulin, GAD65 and IA-2 have been used to predict the risk for development of T1D [115,116] but they are less useful as surrogate markers for monitoring disease progression [118]. In this thesis, we reported 26% of T1D patients had abnormally high Glb1 antibody levels (Figure 6.5)[209]. Almost all individuals with high Glb1 antibodies also demonstrated high T cell responses to Glb1 protein (Figure 6.6). Such a presence of both humoral and Th1 cell mediated immune response is similar to well known responses to microbial pathogens and autoantigens. For example, the presence of both antibody and specific T cells against diabetes autoantigens has been reported in T1D patients [115]. Another observation consistent with this interpretation is the presence of low levels of the Th2 cytokine, IL-4 in T1D patients. Nonetheless, these levels were present at higher concentrations compared with supernatants from cells of control individuals (Figure 3.5).

Potential role of wheat reactive immune cells in T1D pathogenesis

Strong T cell reactivity to WP and Glb1 and antibody responses to Glb1 in a patient with T1D/CD [143] (Chapter 5) and also in a subset of young adult T1D patients [207](Chapter 3), raises the question whether this abnormal immune response can promote beta cell destruction. Alternatively, these immune responses may only reflect abnormalities in the gut immune system or lack of immune tolerance in these patients. Previous studies from our

laboratory showed that antibody reactivity to Glb1 correlated with islet inflammation and damage in BBdp rats [86].

To address the potential role of wheat reactive immune responses to promote beta cell damage, I would like to discuss possible mechanisms that could be involved in wheat-induced immune destruction of beta cells in the pancreas. If we expect a pathogenic role for WP-induced cells in the diabetic process, those cells should be capable of trafficking to PLN and the pancreas and further promote islet endocrine pathology. Some reports indicate that adoptive transfer of immune cells isolated from the MLN of NOD mice and BBdp rats can traffic to the pancreas and promote diabetes [94,210]. In addition, T1D patients displayed autoreactive T cells expressing the gut-associated homing receptor $\alpha4\beta7$ integrin [95]. In our study this homing receptor was expressed on 50-60% of WP-reactive T cells. Although the percentage of $\alpha4\beta7$ was not significantly different between reactive and non reactive T cells (data not shown), this still is an indication that most of wheat reactive T cells in PBMC have a gut homing receptor.

Molecular mimicry is one of the suggested mechanisms as a trigger for autoimmunity [201]. This is a phenomenon whereby some environmental antigens evoke an immune response that cannot distinguish self from non-self antigens, resulting in an autoimmune reaction [211]. There are reports that foreign antigens could accelerate the autoimmune attack on target cells by this process [212]. Some proposed examples of molecular mimicry in T1D and CD are as follows: an epitope in bovine serum albumin in cow milk, termed ABBOS was shown to be similar to the beta cell protein ICA69 [69]. An epitope in NADH ubiquinone from wheat and soybean displays some homology with the T1D autoantigen, IA-2 [213]. Also, recently it was reported that anti-gliadin antibodies in immunized animals and CD patients cross react with

the synapsin I protein [214]. Preliminary results from my studies, and those of my colleagues indicate that Glb1 antibodies generated in rabbit or purified from sera of our index patient with both T1D and coeliac disease are able to bind to 62 KDa protein in human islets. Further study is required to evaluate and characterize the potential role of Glb1 antibodies in mimicry recognition of self-antigen and potential role in development of diabetes.

Another possible mechanism is immune activation of specific autoimmune cells by bystander lymphocyte activation. This mechanism proceeds when the immune response to a non-self antigen causes the activation of autoimmune cells which normally circulate at low frequency in the same micro-environment [215]. This process could occur in the gut immune system in response to WP in genetically predisposed individuals, simply, β cell specific immune cells that normally circulate at very low frequency become activated following changes in the microenvironment of the gut immune system.

Model for involvement of dietary wheat proteins in development of T1D

The data from this thesis are consistent with previous reports suggesting a role for wheat proteins in diabetes-prone animals and humans29. Most of the evidence linking the development of diabetes in human to wheat comes from epidemiological studies. We propose a model in which dietary wheat protein is one possible pathway of disease progression in a subset of T1D patients (Figure 7.1). Based on the general model of environmental modification of gut immune system [34], the effect of environmental agents on genetically predisposed individuals depends on age of exposure and dosage. The combination of these conditions will prevent or promote diabetes pathogenesis. As summarized in the legend of my current model (Figure 7.1), damage to the gut epithelium in some individuals permits entry of excessive amounts of dietary antigens including WP to the

GALT. Specific dietary proteins could promote an immune reaction, which causes secretion of pro-inflammatory cytokines and activation and proliferation of different subtypes of cells including T cells, B-cells and professional antigen presenting cells. One possibility is that the immune response to dietary antigens modifies the immune attack on beta cells in the pancreas either by a direct or bystander mechanism.

The following points are highlights of some novel findings in this study which support such a model.

- In this study we reported more that 40% of T1D patients showed abnormal immune responses to WP and Glb1. The gut is the main entry point for these antigens, therefore the abnormal immune responses which we detected in peripheral blood likely also reflect abnormal immune responses in the gut immune system.
- The finding that WP responders were predominantly HLA-DR4 (>95%) and not HLA-DQ2, supports the interpretation that this effect was diabetes-specific (Chapter 3). This result is similar to that reported by Outi Vaarala *et al.* [96] indicating that intestinal immune activation in T1D was not restricted to patients with the HLA-DQ2 genotype.
- Antibody response to Glb1 was abnormally high in a subset of T1D patients and interestingly both T cell and antibody responses to Glb1 were correlated in this subset (Chapter 6).
- We showed a strong T cell reactivity to WP and Glb1 with a predominant Th1 cytokine pattern in the supernatant of PBMC cultures (Chapter 3 and 6). IL-4 was also increased in response to Glb1 indicating the presence of a Th2 response in parallel with the dominant Th1 response, which may explain in part the presence of a strong IgG reactivity to Glb1 in some of our patients (Chapter 6).

- The high concentrations of IL-6 in the supernatant of WP and Glb1 stimulated cultures were not inhibited by anti-DR antibody, suggesting that IL-6 was secreted from other cell types. The high level of IL-6 produced in response to WP (4-6 fold higher than IFN-γ) further suggests a mechanism by which wheat can promote the development of T1D through induction of powerful pro-inflammatory cytokines.
- The abnormal immune response to Glb1 in the case of a highly wheat sensitive patient presented in chapter 5[143] raised the question whether these responses were associated with T1D or CD.
- The correlation between Glb1 antibody and zonulin in young subjects at high risk for T1D raised the possibility that the presence of this dietary antigen in the context of increased gut permeability induced abnormal immune responses.
- Increased immune responses to Glb1 were also found in children with high genetic risk for diabetes who developed CD autoimmunity. Interestingly, antibody response to Glb1 was observed considerably before the appearance of antibodies to the CD autoantigen, tTG (Chapter 6).

Overall, these data provide evidence that selected dietary antigens could induce abnormal immune responses in some individuals at risk for T1D. One focus of our laboratory is to investigate molecular mimicry as a possible mechanism in T1D pathogenesis. Preliminary results from ongoing research in our laboratory suggest that Glb1 antibodies generated in rabbit or isolated from serum of our index patient can bind to specific proteins in human islets. These intriguing data require confirmation. In order to clarify the proposed model, it is important to investigate trafficking of specific WP or Glb1 T cells to pancreatic lymph nodes and islets. In addition, the possible role of Glb1

antibodies and wheat specific T cells in beta cell function and pathology should be determined.

Despite ongoing research in the field of T1D, the pathogenesis of this disease in human remains unclear and data on environmental interactions are conflicting and sparse. Although, we do not expect that all cases of diabetes are wheat-related, it does appear to be involved in some patients. Identification of a subset of T1D individuals who have abnormal humoral and cellular immune responses to wheat proteins, raises the possibility that these immune responses play a role in the Th1-based insulitis, which can lead to T1D. Further studies are required to determine whether these diet antigen specific T cells and antibodies have direct effects on target tissues such as the gut and pancreas.

Future directions

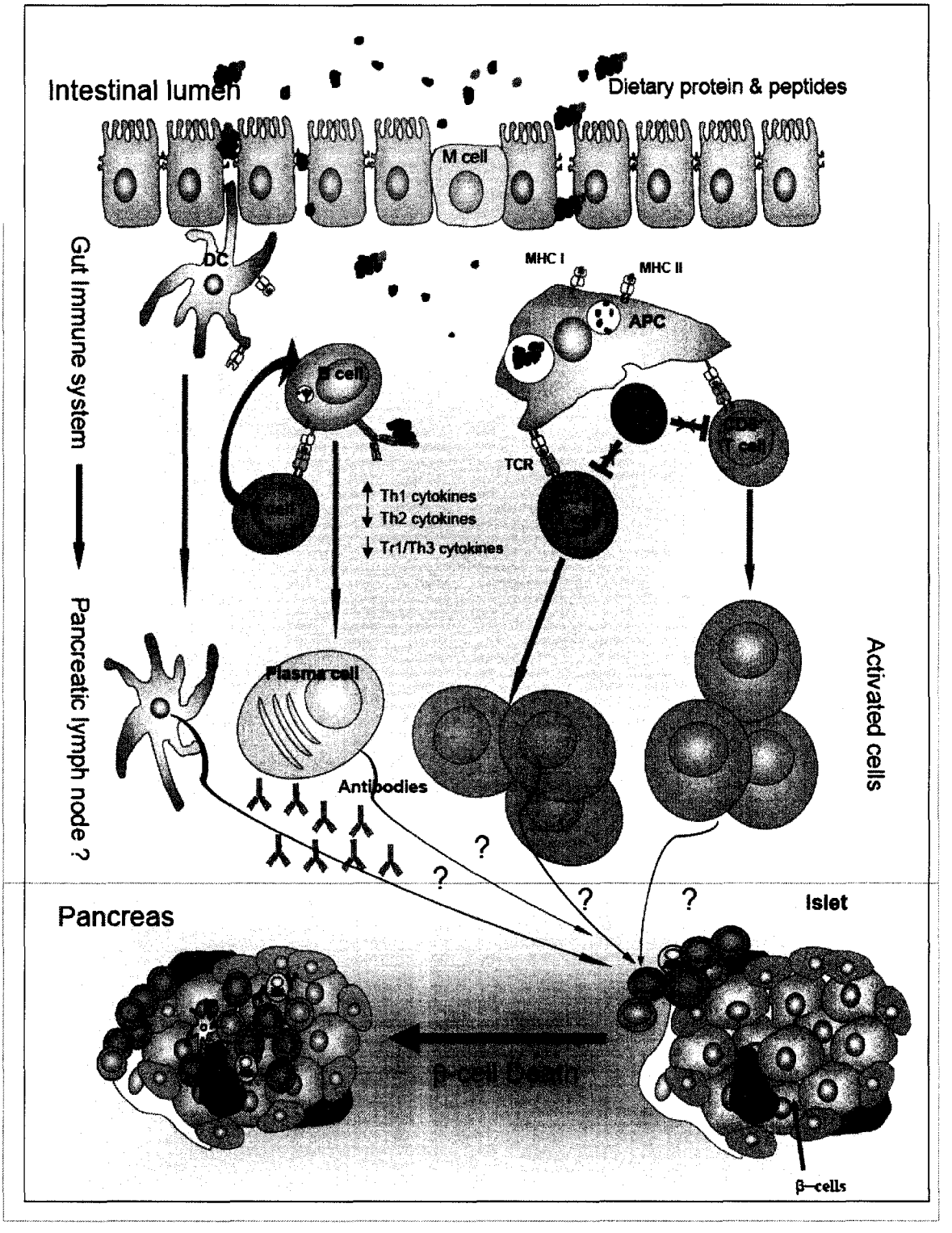
In humans, the link between the gut immune system and T1D has been suggested by several reports [68,87,95,96,216]. Evidence is also accumulating in support of a wheat-T1D link. For example, the coincidence of T1D and coeliac disease has been well documented. In general, in most cases of T1D/CD, coeliac disease is diagnosed after T1D onset. In contrast, in individuals who develop coeliac disease first, the chance of T1D development is lower, suggesting that a gluten free diet, which is the treatment for CD, can prevent the development of diabetes [135,217]. There is additional evidence supporting the gut-wheat-T1D link: (1) long-term exposure to wheat protein was associated with development of T1D [218], (2) antibodies to the endomysial autoantigen, tissue transglutaminase were present in 20-30% of T1D patients [190], (3) the gut associated homing receptor $\alpha 4\beta 7$-integrin was present on autoreactive T cells from T1D patients [95], and (4) intestinal immune activation that was

not HLA-DQ restricted was observed in T1D patients. Therefore, the main approach for future studies should focus on understanding how dietary proteins including wheat, interact with the gut immune system in normal and diabetes-prone individuals and understanding how this process could affect islet biology.

Identification of diabetes promoting environmental antigens, specifically wheat antigens will provide the potential to develop preventive treatments using foods lacking or low in specific antigens; it may also be possible to engineer new cereal products with low diabetes-promoting capacity. It will be important to: (1) expand these studies with more individuals, (2) choose appropriate control subjects to evaluate whether immune responses to Glb1 are a general marker of gut damage or more specifically can be used as a predictor of development of T1D, CD or cases who have both diseases.

Figure 7. 1. A model for involvement of dietary wheat proteins in development of T1D

In genetically predisposed individuals, entry of an excessive amount of dietary antigens, specifically wheat proteins (possibly whole proteins and peptides) occurs via a leaky gut barrier to the lamina propria and the draining mesenteric lymph nodes. Dietary peptides can also pass through the normal route of uptake via the M cells that coat the Peyer's patches. Professional antigen-presenting cells (APC) including dendritic cells, macrophages and B cells take up wheat protein antigens and present the peptides on MHC class II molecules to $CD3^+$ ($CD4^+$ and $CD8^+$) T cells. Wheat activated T cells secrete pro-inflammatory Th1 cytokines and IL-6. Because of the pro-inflammatory state of the gut and the presence of excess dietary antigens, regulatory T cells are less able to suppress the effect of pro-inflammatory Th1 cytokines on T cells, B cells, and APCs. In parallel, B cells sample dietary peptides and present them to previously activated $CD4^+$ T-helper cells. Activated B cells proliferate and produce immunoglobulins. Therefore, these various populations of activated cells expand probably in the draining mesenteric lymph nodes and potentially in the pancreatic lymph nodes. Activated immune cells migrate to the pancreas where they could promote destruction of β-cells.


Intestinal lumen
Dietary protein & peptides
M cell
DC
MHC I
MHC II
APC
B cell
TCR
Th1 cytokines
Th2 cytokines
Tr1/Th3 cytokines
Gut Immune system
Pancreatic lymph node ?
Plasma cell
Antibodies
Activated cells
?
?
?
?
Pancreas
Islet
β-cell death
β-cells

Conclusions

A sensitive CFSE proliferation assay was optimized and adapted to measure $CD3^+$ T cell responses to wheat proteins (chymotrypsin-treated, heat-inactivated wheat gluten) and Glb1 recombinant protein in PBMC of T1D patients (Chapter 2 and 3). Our data are consistent with previous reports linking wheat proteins with diabetes development in diabetes-prone animals and humans. Results from this study indicate that a subset of young adult T1D patients display an abnormal humoral and cellular immune response to Glb1, a dietary wheat protein. T cell responses to WP and Glb1 were associated with the diabetes risk allele HLA-DR (Chapter 3, 6), such that WP-responders were predominantly HLA-DR4, and not HLA-DQ2 (Chapter 3), suggesting that this effect was diabetes-specific. An abnormal immune response to Glb1 was observed for the first time in a human patient who was highly wheat sensitive with both CD and T1D, and this raised the question whether this response was associated with T1D or CD (Chapter 5) [143]. Glb1 antibody and zonulin level were correlated in a subset of young individuals with islet autoimmunity [208]. In children at high genetic risk for diabetes who developed CD autoimmunity, increased Glb1 antibody level was observed before antibodies to the CD autoantigen, tTG [192] appeared. These data further suggest the possibility that gut barrier dysfunction is present in some T1D patients. This could permit entry of unusually high amounts of dietary antigens including Glb1, causing an imbalance in the gut immune system associated with a breakdown of tolerance. Further studies are required to evaluate whether immune responses to Glb1 are a general marker of gut damage or more specifically can be used as a predictor of development of T1D. It also remains to be determined, whether abnormal immunity to Glb1 or other

components of wheat may affect the immune attack on the pancreas by a process that remains to be elucidated.

REFERENCES

1. Tattersall .Robert B. 2003. The history of diabetes mellitus. In *Textbook of diabetes*, Vol. 1. J. C. Pickup and G. Williams, eds. Blackwell Science, p. 1.1-1.22.

2. Gepts, W. 1965. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14:619-633.

3. Rossini, A. A. 2004. Autoimmune diabetes and the circle of tolerance. *Diabetes* 53:267-275.

4. Tuomi, T. 2005. Type 1 and type 2 diabetes: what do they have in common? *Diabetes* 54 Suppl 2:S40-S45.

5. Seissler, J. and W. A. Scherbaum. 2006. Autoimmune diagnostics in diabetes mellitus. *Clin.Chem.Lab Med.* 44:133-137.

6. Alberti, K. G. and P. Z. Zimmet. 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet.Med.* 15:539-553.

7. 2004. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 27 Suppl 1:S5-S10.

8. Gerard Slama. 2003. Type 1 diabetes: an overview. In *Textbook of Diabetes*, Vol. 1. John C.Pickup & Gareth Williams and Gerard Slama, eds. Blackwell Science, p. 5.1-5.14.

9. Devendra, D., E. Liu, and G. S. Eisenbarth. 2004. Type 1 diabetes: recent developments. *BMJ* 328:750-754.

10. Karvonen, M., M. Viik-Kajander, E. Moltchanova, I. Libman, R. LaPorte, and J. Tuomilehto. 2000. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. *Diabetes Care* 23:1516-1526.

11. Casu, A., C. Pascutto, L. Bernardinelli, and M. Songini. 2004. Type 1 diabetes among sardinian children is increasing: the Sardinian diabetes register for children aged 0-14 years (1989-1999). *Diabetes Care* 27:1623-1629.

12. Karvonen, M., J. Pitkaniemi, and J. Tuomilehto. 1999. The onset age of type 1 diabetes in Finnish children has become younger. The Finnish Childhood Diabetes Registry Group. *Diabetes Care* 22:1066-1070.

13. Li, X. H., T. L. Li, Z. Yang, Z. Y. Liu, Y. D. Wei, S. X. Jin, C. Hong, R. L. Qin, Y. Q. Li, J. S. Dorman, R. E. Laporte, and K. A. Wang. 2000. A nine-year prospective

study on the incidence of childhood type 1 diabetes mellitus in China. *Biomed.Environ.Sci.* 13:263-270.

14. Newhook, L. A., J. Curtis, D. Hagerty, M. Grant, A. D. Paterson, C. Crummel, T. Bridger, and P. Parfrey. 2004. High incidence of childhood type 1 diabetes in the Avalon Peninsula, Newfoundland, Canada. *Diabetes Care* 27:885-888.

15. Gale, E. A. 2002. The rise of childhood type 1 diabetes in the 20th century. *Diabetes* 51:3353-3361.

16. Gillespie, K. M., S. C. Bain, A. H. Barnett, P. J. Bingley, M. R. Christie, G. V. Gill, and E. A. Gale. 2004. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. *Lancet* 364:1699-1700.

17. Onkamo, P., S. Vaananen, M. Karvonen, and J. Tuomilehto. 1999. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 42:1395-1403.

18. Knip, M., R. Veijola, S. M. Virtanen, H. Hyoty, O. Vaarala, and H. K. Akerblom. 2005. Environmental triggers and determinants of type 1 diabetes. *Diabetes* 54 Suppl 2:S125-S136.

19. Fathman, C. G., L. Soares, S. M. Chan, and P. J. Utz. 2005. An array of possibilities for the study of autoimmunity. *Nature* 435:605-611.

20. Eisenbarth, G. S. 1986. Type I diabetes mellitus. A chronic autoimmune disease. *N.Engl.J.Med.* 314:1360-1368.

21. Haller, M. J., M. A. Atkinson, and D. Schatz. 2005. Type 1 diabetes mellitus: etiology, presentation, and management. *Pediatr.Clin.North Am.* 52:1553-1578.

22. Hamalainen, A. M. and M. Knip. 2002. Autoimmunity and familial risk of type 1 diabetes. *Curr.Diab.Rep.* 2:347-353.

23. Sheehy, M. J., S. J. Scharf, J. R. Rowe, M. H. Neme de Gimenez, L. M. Meske, H. A. Erlich, and B. S. Nepom. 1989. A diabetes-susceptible HLA haplotype is best defined by a combination of HLA-DR and -DQ alleles. *J.Clin.Invest* 83:830-835.

24. Ueda, H., J. M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D. B, et al. 2003. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423:506-511.

25. Bohren, K. M., V. Nadkarni, J. H. Song, K. H. Gabbay, and D. Owerbach. 2004. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J.Biol.Chem.* 279:27233-27238.

26. Sasaki, Y., K. Ihara, N. Matsuura, H. Kohno, S. Nagafuchi, R. Kuromaru, K. Kusuhara, R. Takeya, T. Hoey, H. Sumimoto, and T. Hara. 2004. Identification of a novel type 1 diabetes susceptibility gene, T-bet. *Hum.Genet.* 115:177-184.

27. Park, Y., S. Park, E. Yoo, D. Kim, and H. Shin. 2004. Association of the polymorphism for Toll-like receptor 2 with type 1 diabetes susceptibility. *Ann.N.Y.Acad.Sci.* 1037:170-174.

28. Yokohama. Allele frequencies. The central Data analysis committe, Section 6. 3. Splits combined five loci. 2, 807-14. 1991. Ref Type: Conference Proceeding

29. MacFarlane, A. J. and F. W. Scott. 2003. Environmental agents and type 1 diabetes. In *Texbook of diabetes 1*. J. C. Pickup and G. Williams, eds. Blackwell publishing, p. 17.1-17.16.

30. Field, L. L. 2002. Genetic linkage and association studies of Type I diabetes: challenges and rewards. *Diabetologia* 45:21-35.

31. Gianani, R. and G. S. Eisenbarth. 2005. The stages of type 1A diabetes: 2005. *Immunol.Rev.* 204:232-249.

32. Jahromi, M. M. and G. S. Eisenbarth. 2006. Genetic determinants of type 1 diabetes across populations. *Ann.N.Y.Acad.Sci.* 1079:289-299.

33. Sanjeevi, C. B., C. DeWeese, M. Landin-Olsson, I. Kockum, G. Dahlquist, A. Lernmark, and T. P. Lybrand. 1997. Analysis of critical residues of HLA-DQ6 molecules in insulin-dependent diabetes mellitus. *Tissue Antigens* 50:61-65.

34. Lefebvre, D. E., K. L. Powell, A. Strom, and F. W. Scott. 2006. Dietary proteins as environmental modifiers of type 1 diabetes mellitus. *Annu.Rev.Nutr.* 26:175-202.

35. Pugliese, A. 2004. Genetics of type 1 diabetes. *Endocrinol.Metab Clin.North Am.* 33:1-16, vii.

36. Onkamo, P., S. Vaananen, M. Karvonen, and J. Tuomilehto. 1999. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 42:1395-1403.

37. Kondrashova, A., A. Reunanen, A. Romanov, A. Karvonen, H. Viskari, T. Vesikari, J. Ilonen, M. Knip, and H. Hyoty. 2005. A six-fold gradient in the incidence of type 1 diabetes at the eastern border of Finland. *Ann.Med.* 37:67-72.

38. Graves, P. M., J. M. Norris, M. A. Pallansch, I. C. Gerling, and M. Rewers. 1997. The role of enteroviral infections in the development of IDDM: limitations of current approaches. *Diabetes* 46:161-168.

39. Menser, M. A., J. M. Forrest, and R. D. Bransby. 1978. Rubella infection and diabetes mellitus. *Lancet* 1:57-60.

40. Forrest, J. M., M. A. Menser, and J. A. Burgess. 1971. High frequency of diabetes mellitus in young adults with congenital rubella. *Lancet* 2:332-334.

41. Karounos, D. G., J. S. Wolinsky, and J. W. Thomas. 1993. Monoclonal antibody to rubella virus capsid protein recognizes a beta-cell antigen. *J.Immunol.* 150:3080-3085.

42. Bodansky, H. J., P. J. Grant, B. M. Dean, J. McNally, G. F. Bottazzo, M. H. Hambling, and J. K. Wales. 1986. Islet-cell antibodies and insulin autoantibodies in association with common viral infections. *Lancet* 2:1351-1353.

43. Ginsberg-Fellner, F., M. E. Witt, S. Yagihashi, M. J. Dobersen, F. Taub, B. Fedun, R. C. McEvoy, S. H. Roman, R. G. Davies, L. Z. Cooper, and . 1984. Congenital rubella syndrome as a model for type 1 (insulin-dependent) diabetes mellitus: increased prevalence of islet cell surface antibodies. *Diabetologia* 27 Suppl:87-89.

44. Clements, G. B., D. N. Galbraith, and K. W. Taylor. 1995. Coxsackie B virus infection and onset of childhood diabetes. *Lancet* 346:221-223.

45. Hyoty, H., M. Hiltunen, M. Knip, M. Laakkonen, P. Vahasalo, J. Karjalainen, P. Koskela, M. Roivainen, P. Leinikki, T. Hovi, and . 1995. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes* 44:652-657.

46. Dahlquist, G. G., S. Ivarsson, B. Lindberg, and M. Forsgren. 1995. Maternal enteroviral infection during pregnancy as a risk factor for childhood IDDM. A population-based case-control study. *Diabetes* 44:408-413.

47. Banatvala, J. E. 1987. Insulin-dependent (juvenile-onset, type 1) diabetes mellitus Coxsackie B viruses revisited. *Prog.Med.Virol.* 34:33-54.

48. Frisk, G., E. Nilsson, T. Tuvemo, G. Friman, and H. Diderholm. 1992. The possible role of Coxsackie A and echo viruses in the pathogenesis of type I diabetes mellitus studied by IgM analysis. *J.Infect.* 24:13-22.

49. Szopa, T. M., T. Ward, D. M. Dronfield, N. D. Portwood, and K. W. Taylor. 1990. Coxsackie B4 viruses with the potential to damage beta cells of the islets are present in clinical isolates. *Diabetologia* 33:325-328.

50. Green, J., D. Casabonne, and R. Newton. 2004. Coxsackie B virus serology and Type 1 diabetes mellitus: a systematic review of published case-control studies. *Diabet.Med.* 21:507-514.

51. Gerling, I., N. K. Chatterjee, and C. Nejman. 1991. Coxsackievirus B4-induced development of antibodies to 64,000-Mr islet autoantigen and hyperglycemia in mice. *Autoimmunity* 10:49-56.

52. Graves, P. M., H. A. Rotbart, W. A. Nix, M. A. Pallansch, H. A. Erlich, J. M. Norris, M. Hoffman, G. S. Eisenbarth, and M. Rewers. 2003. Prospective study of enteroviral infections and development of beta-cell autoimmunity. Diabetes autoimmunity study in the young (DAISY). *Diabetes Res.Clin.Pract.* 59:51-61.

53. Fuchtenbusch, M., A. Irnstetter, G. Jager, and A. G. Ziegler. 2001. No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes. *J.Autoimmun.* 17:333-340.

54. Myers, M. A., K. D. Hettiarachchi, J. P. Ludeman, A. J. Wilson, C. R. Wilson, and P. Z. Zimmet. 2003. Dietary microbial toxins and type 1 diabetes. *Ann.N.Y.Acad.Sci.* 1005:418-422.

55. Vaarala, O. 1999. Gut and the induction of immune tolerance in type 1 diabetes. *Diabetes Metab Res.Rev.* 15:353-361.

56. Rossini, A. A., R. M. Williams, J. P. Mordes, M. C. Appel, and A. A. Like. 1979. Spontaneous diabetes in the gnotobiotic BB/W rat. *Diabetes* 28:1031-1032.

57. Lefebvre D.E., Wang G, Patrick C., Crookshank J.A, and Scott f.w. Differentiating the role of microbes and diet in development of autoimmune diabetes. Diabetes Diabetes, June Supplement, 67th Scientific Sessions- Annual Meeting of American Diabetes Association(67th Scientific Sessions (ADA)). 1-6-2007. Ref Type: Abstract

58. Akerblom, H. K. and M. Knip. 1998. Putative environmental factors in Type 1 diabetes. *Diabetes Metab Rev* 14:31-67.

59. Elliott, R. B. and J. M. Martin. 1984. Dietary protein: a trigger of insulin-dependent diabetes in the BB rat? *Diabetologia* 26:297-9.

60. Coleman, D. L., J. E. Kuzava, and E. H. Leiter. 1990. Effect of diet on incidence of diabetes in nonobese diabetic mice. *Diabetes* 39:432-6.

61. Scott, F. W. 1996. Food-induced type 1 diabetes in the BB rat. *Diabetes Metab Rev* 12:341-59.

62. Gerstein, H. C. 1994. Cow's milk exposure and type I diabetes mellitus. A critical overview of the clinical literature. *Diabetes Care* 17:13-19.

63. Scott, F. W., J. M. Norris, and H. Kolb. 1996. Milk and type I diabetes. *Diabetes Care* 19:379-383.

64. Wasmuth, H. E. and H. Kolb. 2000. Cow's milk and immune-mediated diabetes. *Proc.Nutr.Soc.* 59:573-579.

65. Bodington, M. J., P. G. McNally, and A. C. Burden. 1994. Cow's milk and type 1 childhood diabetes: no increase in risk. *Diabet.Med.* 11:663-665.

66. Karisson, M. G., J. Garcia, and J. Ludvigsson. 2001. Cows' milk proteins cause similar Th1- and Th2-like immune response in diabetic and healthy children. *Diabetologia* 44:1140-1147.

67. Schatz, D. A. and N. K. Maclaren. 1996. Cow's milk and insulin-dependent diabetes mellitus. Innocent until proven guilty. *Jama* 276:647-648.

68. Akerblom, H. K., S. M. Virtanen, J. Ilonen, E. Savilahti, O. Vaarala, A. Reunanen, K. Teramo, A. M. Hamalainen, J. Paronen, M. A. Riikjarv, A. Ormisson, J. Ludvigsson, H. M. Dosch, T. Hakulinen, and M. Knip. 2005. Dietary manipulation of beta cell autoimmunity in infants at increased risk of type 1 diabetes: a pilot study. *Diabetologia* 48:829-837.

69. Karjalainen, J., J. M. Martin, M. Knip, J. Ilonen, B. H. Robinson, E. Savilahti, H. K. Akerblom, and H. M. Dosch. 1992. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N.Engl.J.Med.* 327:302-307.

70. Sarugeri, E., N. Dozio, F. Meschi, M. R. Pastore, and E. Bonifacio. 1999. Cellular and humoral immunity against cow's milk proteins in type 1 diabetes. *J.Autoimmun.* 13:365-373.

71. Atkinson, M. A., M. A. Bowman, K. J. Kao, L. Campbell, P. J. Dush, S. C. Shah, O. Simell, and N. K. Maclaren. 1993. Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes. *N.Engl.J.Med.* 329:1853-1858.

72. Hummel, M., M. Fuchtenbusch, M. Schenker, and A. G. Ziegler. 2000. No major association of breast-feeding, vaccinations, and childhood viral diseases with early islet autoimmunity in the German BABYDIAB Study. *Diabetes Care* 23:969-974.

73. Couper, J. J., C. Steele, S. Beresford, T. Powell, K. McCaul, A. Pollard, S. Gellert, B. Tait, L. C. Harrison, and P. G. Colman. 1999. Lack of association between duration of breast-feeding or introduction of cow's milk and development of islet autoimmunity. *Diabetes* 48:2145-2149.

74. Wright, A. L. 2001. The rise of breastfeeding in the United States. *Pediatr.Clin.North Am.* 48:1-12.

75. Consultative Group on International Agricultural Research (CGIAR). WHEAT IS CENTURY'S 'MIRACLE CROP'. Consultative Group on International Agricultural Research . 1995.

76. Gianibelli MC and Larroque OR, MacRitchieF Wrigley CW. Biochemical, genetic, and molecular characterization of wheat endosperm proteins. Am.Assoc.Cereal Chemist Publ.No.C-2001-0926-010 Ref Type: Electronic Citation http://www.aaccnet.org/cerealchemistry/freearticle/gianibelli.pdf. 2001.

77. Hischenhuber, C., R. Crevel, B. Jarry, M. Maki, D. A. Moneret-Vautrin, A. Romano, R. Troncone, and R. Ward. 2006. Review article: safe amounts of gluten for patients with wheat allergy or coeliac disease. *Aliment.Pharmacol.Ther.* 23:559-575.

78. Brisman, J. 2002. Baker's asthma. *Occup.Environ.Med.* 59:498-502.

79. Scott, F. W., H. E. Cloutier, R. Kleemann, U. Woerz-Pagenstert, P. Rowsell, H. W. Modler, and H. Kolb. 1997. Potential mechanisms by which certain foods promote or inhibit the development of spontaneous diabetes in BB rats: dose, timing, early effect on islet area, and switch in infiltrate from Th1 to Th2 cells. *Diabetes* 46:589-98.

80. Scott, F. W., G. Sarwar, and H. E. Cloutier. 1988. Diabetogenicity of various protein sources in the diet of the diabetes-prone BB rat. *Adv Exp Med Biol* 246:277-85.

81. Beales, P. E., R. B. Elliott, S. Flohe, J. P. Hill, H. Kolb, P. Pozzilli, G. S. Wang, H. Wasmuth, and F. W. Scott. 2002. A multi-centre, blinded international trial of the effect of A(1) and A(2) beta-casein variants on diabetes incidence in two rodent models of spontaneous Type I diabetes. *Diabetologia* 45:1240-6.

82. Karges, W., D. Hammond-McKibben, R. K. Cheung, M. Visconti, N. Shibuya, D. Kemp, and H. M. Dosch. 1997. Immunological aspects of nutritional diabetes prevention in NOD mice: a pilot study for the cow's milk-based IDDM prevention trial. *Diabetes* 46:557-64.

83. Rensch, M. J., J. A. Merenich, M. Lieberman, B. D. Long, D. R. Davis, and P. R. McNally. 1996. Gluten-sensitive enteropathy in patients with insulin-dependent diabetes mellitus. *Ann Intern Med* 124:564-7.

84. Saukkonen, T., E. Savilahti, H. Reijonen, J. Ilonen, E. Tuomilehto-Wolf, and H. K. Akerblom. 1996. Coeliac disease: frequent occurrence after clinical onset of insulin-dependent diabetes mellitus. Childhood Diabetes in Finland Study Group. *Diabet Med* 13:464-70.

85. Klemetti, P., E. Savilahti, J. Ilonen, H. K. Akerblom, and O. Vaarala. 1998. T-cell reactivity to wheat gluten in patients with insulin-dependent diabetes mellitus. *Scand J Immunol* 47:48-53.

86. MacFarlane, A. J., K. M. Burghardt, J. Kelly, T. Simell, O. Simell, I. Altosaar, and F. W. Scott. 2003. A type 1 diabetes-related protein from wheat (Triticum aestivum). cDNA clone of a wheat storage globulin, Glb1, linked to islet damage. *J Biol Chem* 278:54-63.

87. Ziegler, A. G., S. Schmid, D. Huber, M. Hummel, and E. Bonifacio. 2003. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. *Jama* 290:1721-8.

88. Norris, J. M., K. Barriga, G. Klingensmith, M. Hoffman, G. S. Eisenbarth, H. A. Erlich, and M. Rewers. 2003. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. *Jama* 290:1713-20.

89. Pastore, M. R., E. Bazzigaluppi, C. Belloni, C. Arcovio, E. Bonifacio, and E. Bosi. 2003. Six months of gluten-free diet do not influence autoantibody titers, but improve insulin secretion in subjects at high risk for type 1 diabetes. *J.Clin.Endocrinol.Metab* 88:162-165.

90. Tlaskalova-Hogenova, H., R. Stepankova, T. Hudcovic, L. Tuckova, B. Cukrowska, R. Lodinova-Zadnikova, H. Kozakova, P. Rossmann, J. Bartova, D. Sokol, D. P. Funda, D. Borovska, Z. Rehakova, J. Sinkora, J. Hofman, P. Drastich, and A. Kokesova. 2004. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol.Lett.* 93:97-108.

91. Elliott, R. B., S. N. Reddy, N. J. Bibby, and K. Kida. 1988. Dietary prevention of diabetes in the non-obese diabetic mouse. *Diabetologia* 31:62-4.

92. Hanninen, A., M. Salmi, O. Simell, and S. Jalkanen. 1996. Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior. *Diabetes* 45:1173-1180.

93. Yang, X. D., H. K. Sytwu, H. O. McDevitt, and S. A. Michie. 1997. Involvement of beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in the development of diabetes in obese diabetic mice. *Diabetes* 46:1542-7.

94. Hanninen, A., I. Jaakkola, and S. Jalkanen. 1998. Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J.Immunol.* 160:6018-6025.

95. Paronen, J., P. Klemetti, J. M. Kantele, E. Savilahti, J. Perheentupa, H. K. Akerblom, and O. Vaarala. 1997. Glutamate decarboxylase-reactive peripheral blood lymphocytes from patients with IDDM express gut-specific homing receptor alpha4beta7-integrin. *Diabetes* 46:583-8.

96. Vaarala, O. 2004. Intestinal immunity and type 1 diabetes. *J.Pediatr.Gastroenterol.Nutr.* 39 Suppl 3:S732-S733.

97. Carratu, R., M. Secondulfo, L. de Magistris, D. Iafusco, A. Urio, M. G. Carbone, G. Pontoni, M. Carteni, and F. Prisco. 1999. Altered intestinal permeability to mannitol in diabetes mellitus type I. *J Pediatr Gastroenterol Nutr* 28:264-9.

98. Damci, T., I. Nuhoglu, G. Devranoglu, Z. Osar, M. Demir, and H. Ilkova. 2003. Increased intestinal permeability as a cause of fluctuating postprandial blood glucose levels in Type 1 diabetic patients. *Eur J Clin Invest* 33:397-401.

99. Mooradian, A. D., J. E. Morley, A. S. Levine, W. F. Prigge, and R. L. Gebhard. 1986. Abnormal intestinal permeability to sugars in diabetes mellitus. *Diabetologia* 29:221-224.

100. Secondulfo, M., D. Iafusco, R. Carratu, L. deMagistris, A. Sapone, M. Generoso, A. Mezzogiomo, F. C. Sasso, M. Carteni, R. De Rosa, F. Prisco, and V. Esposito. 2004. Ultrastructural mucosal alterations and increased intestinal permeability in non-celiac, type I diabetic patients. *Dig Liver Dis* 36:35-45.

101. Watts, T., I. Berti, A. Sapone, T. Gerarduzzi, T. Not, R. Zielke, and A. Fasano. 2005. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. *Proc.Natl.Acad.Sci.U.S.A* 102:2916-2921.

102. Malaisse, W. J., P. Courtois, and F. W. Scott. 2004. Insulin-dependent diabetes and gut dysfunction: the BB rat model. *Horm Metab Res* 36:585-94.

103. Courtois, P., A. Sener, F. W. Scott, and W. J. Malaisse. 2004. Peroxidase activity in the intestinal tract of Wistar-Furth, BBc and BBdp rats. *Diabetes Metab Res Rev* 20:305-14.

104. Yoon, J. W. and H. S. Jun. 2005. Autoimmune destruction of pancreatic beta cells. *Am.J.Ther.* 12:580-591.

105. Tisch, R. and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell* 85:291-297.

106. Roep, B. O. 2003. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. *Diabetologia* 46:305-321.

107. Jun, H. S., C. S. Yoon, L. Zbytnuik, N. van Rooijen, and J. W. Yoon. 1999. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J.Exp.Med.* 189:347-358.

108. Corbett, J. A., J. L. Wang, M. A. Sweetland, J. R. Lancaster, Jr., and M. L. McDaniel. 1992. Interleukin 1 beta induces the formation of nitric oxide by beta-cells purified from rodent islets of Langerhans. Evidence for the beta-cell as a source and site of action of nitric oxide. *J.Clin.Invest* 90:2384-2391.

109. Pankewycz, O. G., J. X. Guan, and J. F. Benedict. 1995. Cytokines as mediators of autoimmune diabetes and diabetic complications. *Endocr.Rev.* 16:164-176.

110. Pukel, C., H. Baquerizo, and A. Rabinovitch. 1988. Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. *Diabetes* 37:133-136.

111. Itoh, N., A. Imagawa, T. Hanafusa, M. Waguri, K. Yamamoto, H. Iwahashi, M. Moriwaki, H. Nakajima, J. Miyagawa, M. Namba, S. Makino, S. Nagata, N. Kono, and Y. Matsuzawa. 1997. Requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice. *J.Exp.Med.* 186:613-618.

112. Lampeter, E. F., M. Homberg, K. Quabeck, U. W. Schaefer, P. Wernet, J. Bertrams, H. Grosse-Wilde, F. A. Gries, and H. Kolb. 1993. Transfer of insulin-dependent

diabetes between HLA-identical siblings by bone marrow transplantation. *Lancet* 341:1243-1244.

113. Roep, B. O., S. D. Arden, R. R. de Vries, and J. C. Hutton. 1990. T-cell clones from a type-1 diabetes patient respond to insulin secretory granule proteins. *Nature* 345:632-634.

114. Carter, R. H. 2006. B cells in health and disease. *Mayo Clin.Proc.* 81:377-384.

115. Lieberman, S. M. and T. P. DiLorenzo. 2003. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. *Tissue Antigens* 62:359-377.

116. Palmer, J. P., G. A. Fleming, C. J. Greenbaum, K. C. Herold, L. D. Jansa, H. Kolb, J. M. Lachin, K. S. Polonsky, P. Pozzilli, J. S. Skyler, and M. W. Steffes. 2004. C-peptide is the appropriate outcome measure for type 1 diabetes clinical trials to preserve beta-cell function: report of an ADA workshop, 21-22 October 2001. *Diabetes* 53:250-264.

117. Verge, C. F., R. Gianani, E. Kawasaki, L. Yu, M. Pietropaolo, R. A. Jackson, H. P. Chase, and G. S. Eisenbarth. 1996. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 45:926-933.

118. van de, L. P. and B. O. Roep. 2005. T-cell assays to determine disease activity and clinical efficacy of immune therapy in type 1 diabetes. *Am.J.Ther.* 12:573-579.

119. Roep, B. O. 2002. Autoreactive T cells in endocrine/organ-specific autoimmunity: why has progress been so slow? *Springer Semin.Immunopathol.* 24:261-271.

120. Roep, B. O., G. Duinkerken, G. M. Schreuder, H. Kolb, R. R. de Vries, and S. Martin. 1996. HLA-associated inverse correlation between T cell and antibody responsiveness to islet autoantigen in recent-onset insulin-dependent diabetes mellitus. *Eur.J.Immunol.* 26:1285-1289.

121. Roep, B. O., A. A. Kallan, G. Duinkerken, S. D. Arden, J. C. Hutton, G. J. Bruining, and R. R. de Vries. 1995. T-cell reactivity to beta-cell membrane antigens associated with beta-cell destruction in IDDM. *Diabetes* 44:278-283.

122. Mannering, S. I., J. S. Morris, K. P. Jensen, A. W. Purcell, M. C. Honeyman, P. M. van Endert, and L. C. Harrison. 2003. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. *J Immunol Methods* 283:173-83.

123. Dube, C., A. Rostom, R. Sy, A. Cranney, N. Saloojee, C. Garritty, M. Sampson, L. Zhang, F. Yazdi, V. Mamaladze, I. Pan, J. Macneil, D. Mack, D. Patel, and D. Moher. 2005. The prevalence of celiac disease in average-risk and at-risk Western European populations: a systematic review. *Gastroenterology* 128:S57-S67.

124. Catassi, C. and A. Fasano. 2002. New developments in childhood celiac disease. *Curr.Gastroenterol.Rep.* 4:238-243.

125. Helms, S. 2005. Celiac disease and gluten-associated diseases. *Altern.Med.Rev.* 10:172-192.

126. Mowat, A. M. 2003. Coeliac disease--a meeting point for genetics, immunology, and protein chemistry. *Lancet* 361:1290-1292.

127. Holmes, G. K. 2001. Coeliac disease and Type 1 diabetes mellitus - the case for screening. *Diabet.Med.* 18:169-177.

128. Fraser-Reynolds, K. A., J. D. Butzner, D. K. Stephure, R. A. Trussell, and R. B. Scott. 1998. Use of immunoglobulin A-antiendomysial antibody to screen for celiac disease in North American children with type 1 diabetes. *Diabetes Care* 21:1985-1989.

129. Savilahti, E., O. Simell, S. Koskimies, A. Rilva, and H. K. Akerblom. 1986. Celiac disease in insulin-dependent diabetes mellitus. *J.Pediatr.* 108:690-693.

130. Carlsson, A. K., I. E. Axelsson, S. K. Borulf, A. C. Bredberg, B. A. Lindberg, K. G. Sjoberg, and S. A. Ivarsson. 1999. Prevalence of IgA-antiendomysium and IgA-antigliadin autoantibodies at diagnosis of insulin-dependent diabetes mellitus in Swedish children and adolescents. *Pediatrics* 103:1248-1252.

131. Koletzko, S., A. Burgin-Wolff, B. Koletzko, M. Knapp, W. Burger, D. Gruneklee, G. Herz, W. Ruch, A. Thon, U. Wendel, and . 1988. Prevalence of coeliac disease in diabetic children and adolescents. A multicentre study. *Eur.J.Pediatr.* 148:113-117.

132. Boudraa, G., W. Hachelaf, M. Benbouabdellah, M. Belkadi, F. Z. Benmansour, and M. Touhami. 1996. Prevalence of coeliac disease in diabetic children and their first-degree relatives in west Algeria: screening with serological markers. *Acta Paediatr.Suppl* 412:58-60.

133. Hansen, D., B. Brock-Jacobsen, E. Lund, C. Bjorn, L. P. Hansen, C. Nielsen, C. Fenger, S. T. Lillevang, and S. Husby. 2006. Clinical benefit of a gluten-free diet in type 1 diabetic children with screening-detected celiac disease: a population-based screening study with 2 years' follow-up. *Diabetes Care* 29:2452-2456.

134. Galli-Tsinopoulou, A., S. Nousia-Arvanitakis, D. Dracoulacos, M. Xefteri, and M. Karamouzis. 1999. Autoantibodies predicting diabetes mellitus type I in celiac disease. *Horm.Res.* 52:119-124.

135. Cronin, C. C., A. Feighery, J. B. Ferriss, C. Liddy, F. Shanahan, and C. Feighery. 1997. High prevalence of celiac disease among patients with insulin-dependent (type I) diabetes mellitus. *Am.J.Gastroenterol.* 92:2210-2212.

136. Pocecco, M. and A. Ventura. 1995. Coeliac disease and insulin-dependent diabetes mellitus: a causal association? *Acta Paediatr.* 84:1432-1433.

137. Sapone, A., M. L. de, M. Pietzak, M. G. Clemente, A. Tripathi, F. Cucca, R. Lampis, D. Kryszak, M. Carteni, M. Generoso, D. Iafusco, F. Prisco, F. Laghi, G. Riegler, R. Carratu, D. Counts, and A. Fasano. 2006. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 55:1443-1449.

138. Chakir, H., D. E. Lefebvre, H. Wang, E. Caraher, and F. W. Scott. 2005. Wheat protein-induced proinflammatory T helper 1 bias in mesenteric lymph nodes of young diabetes-prone rats. *Diabetologia* 48:1576-1584.

139. Arentz-Hansen, E. H., S. N. McAdam, O. Molberg, C. Kristiansen, and L. M. Sollid. 2000. Production of a panel of recombinant gliadins for the characterisation of T cell reactivity in coeliac disease. *Gut* 46:46-51.

140. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* 72:248-254.

141. Dziarski, R. 1982. Studies on the mechanism of peptidoglycan- and lipopolysaccharide-induced polyclonal activation. *Infect.Immun.* 35:507-514.

142. Kelemen, K., P. A. Gottlieb, A. L. Putnam, H. W. Davidson, D. R. Wegmann, and J. C. Hutton. 2004. HLA-DQ8-associated T cell responses to the diabetes autoantigen phogrin (IA-2 beta) in human prediabetes. *J.Immunol.* 172:3955-3962.

143. Mojibian, M., H. Chakir, A. J. MacFarlane, D. E. Lefebvre, J. R. Webb, C. Touchie, J. Karsh, J. A. Crookshank, and F. W. Scott. 2006. Immune reactivity to a glb1 homologue in a highly wheat-sensitive patient with type 1 diabetes and celiac disease. *Diabetes Care* 29:1108-1110.

144. Meddings, J. B., J. Jarand, S. J. Urbanski, J. Hardin, and D. G. Gall. 1999. Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. *Am J Physiol* 276:951-7.

145. Arrieta, M. C., L. Bistritz, and J. B. Meddings. 2006. Alterations in intestinal permeability. *Gut* 55:1512-1520.

146. Jahromi, M. M. and G. S. Eisenbarth. 2007. Cellular and molecular pathogenesis of type 1A diabetes. *Cell Mol.Life Sci.* 64:865-872.

147. Onengut-Gumuscu, S. and P. Concannon. 2002. Mapping genes for autoimmunity in humans: type 1 diabetes as a model. *Immunol Rev* 190:182-94.

148. Howdle, P. D. 2006. Gliadin, glutenin or both? The search for the Holy Grail in coeliac disease. *Eur.J.Gastroenterol.Hepatol.* 18:703-706.

149. Flohe, S. B., H. E. Wasmuth, J. B. Kerad, P. E. Beales, P. Pozzilli, R. B. Elliott, J. P. Hill, F. W. Scott, and H. Kolb. 2003. A wheat-based, diabetes-promoting diet induces a Th1-type cytokine bias in the gut of NOD mice. *Cytokine* 21:149-154.

150. Oliver, K. G., J. R. Kettman, and R. J. Fulton. 1998. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin.Chem.* 44:2057-2060.

151. Lindsay, G. K., P. F. Roslansky, and T. J. Novitsky. 1989. Single-step, chromogenic Limulus amebocyte lysate assay for endotoxin. *J.Clin.Microbiol.* 27:947-951.

152. Mattern, T., A. Thanhauser, N. Reiling, K. M. Toellner, M. Duchrow, S. Kusumoto, E. T. Rietschel, M. Ernst, H. Brade, H. D. Flad, and . 1994. Endotoxin and lipid A stimulate proliferation of human T cells in the presence of autologous monocytes. *J.Immunol.* 153:2996-3004.

153. Srimal, S., N. Surolia, S. Balasubramanian, and A. Surolia. 1996. Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A. *Biochem.J.* 315 (Pt 2):679-686.

154. Svejgaard, A., P. Platz, and L. P. Ryder. 1983. HLA and disease 1982--a survey. *Immunol Rev* 70:193-218.

155. Sikora, K., B. S. Anand, S. C. Truelove, P. J. Ciclitira, and R. E. Offord. 1976. Stimulation of lymphocytes from patients with coeliac disease by a subfraction of gluten. *Lancet* 2:389-91.

156. Baker, B. S., J. J. Garioch, S. Bokth, H. Thomas, M. M. Walker, J. N. Leonard, and L. Fry. 1995. Lack of proliferative response by gluten-specific T cells in the blood and gut of patients with dermatitis herpetiformis. *J Autoimmun* 8:561-74.

157. Jensen, K., L. M. Sollid, H. Scott, G. Paulsen, K. Kett, E. Thorsby, and K. E. Lundin. 1995. Gliadin-specific T cell responses in peripheral blood of healthy individuals involve T cells restricted by the coeliac disease associated DQ2 heterodimer. *Scand J Immunol* 42:166-70.

158. Molberg Q, McAdam S.N, Lundin K.E.A, and Sollid M. *Celiac Disease: Methods and Protocols (Studies of Gliadin-Specific T Cells in Celiac Disease)*, Vol. 41. Humana Press Inc., Totowa, NJ, pp. 105-125.

159. Elli, L., E. Dolfini, and M. T. Bardella. 2003. Gliadin cytotoxicity and in vitro cell cultures. *Toxicol Lett* 146:1-8.

160. Gjertsen, H. A., L. M. Sollid, J. Ek, E. Thorsby, and K. E. Lundin. 1994. T cells from the peripheral blood of coeliac disease patients recognize gluten antigens when presented by HLA-DR, -DQ, or -DP molecules. *Scand J Immunol* 39:567-74.

161. Renno, T., A. Attinger, S. Locatelli, T. Bakker, S. Vacheron, and H. R. MacDonald. 1999. Cutting edge: apoptosis of superantigen-activated T cells occurs preferentially after a discrete number of cell divisions in vivo. *J.Immunol.* 162:6312-6315.

162. Lyons, A. B. 2000. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J.Immunol.Methods* 243:147-154.

163. Imagawa, A., T. Hanafusa, S. Tamura, M. Moriwaki, N. Itoh, K. Yamamoto, H. Iwahashi, K. Yamagata, M. Waguri, T. Nanmo, S. Uno, H. Nakajima, M. Namba, S. Kawata, J. I. Miyagawa, and Y. Matsuzawa. 2001. Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close correlation between serological markers and histological evidence of cellular autoimmunity. *Diabetes* 50:1269-1273.

164. Kolb, H., U. Worz-Pagenstert, R. Kleemann, H. Rothe, P. Rowsell, and F. W. Scott. 1996. Cytokine gene expression in the BB rat pancreas: natural course and impact of bacterial vaccines. *Diabetologia* 39:1448-54.

165. Rabinovitch, A., W. Suarez-Pinzon, A. El-Sheikh, O. Sorensen, and R. F. Power. 1996. Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: expression of Th1 cytokines correlates with beta-cell destructive insulitis and IDDM. *Diabetes* 45:749-54.

166. Zipris, D., D. L. Greiner, S. Malkani, B. Whalen, J. P. Mordes, and A. A. Rossini. 1996. Cytokine gene expression in islets and thyroids of BB rats. IFN-gamma and IL-12p40 mRNA increase with age in both diabetic and insulin-treated nondiabetic BB rats. *J Immunol* 156:1315-21.

167. Graham, S., P. Courtois, W. J. Malaisse, J. Rozing, F. W. Scott, and A. M. Mowat. 2004. Enteropathy precedes type 1 diabetes in the BB rat. *Gut* 53:1437-44.

168. Campbell, I. L., M. V. Hobbs, J. Dockter, M. B. Oldstone, and J. Allison. 1994. Islet inflammation and hyperplasia induced by the pancreatic islet-specific overexpression of interleukin-6 in transgenic mice. *Am.J.Pathol.* 145:157-166.

169. Pilstrom, B., L. Bjork, and J. Bohme. 1995. Demonstration of a TH1 cytokine profile in the late phase of NOD insulitis. *Cytokine* 7:806-814.

170. Rabinovitch, A. 1998. An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus. *Diabetes Metab Rev.* 14:129-151.

171. Campbell, I. L., T. W. Kay, L. Oxbrow, and L. C. Harrison. 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J.Clin.Invest* 87:739-742.

172. Ishihara, K. and T. Hirano. 2002. IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev.* 13:357-368.

173. Schuppan, D. and E. G. Hahn. 2001. Celiac disease and its link to type 1 diabetes mellitus. *J Pediatr Endocrinol Metab* 14 Suppl 1:597-605.

174. Margaritte-Jeannin, P., M. C. Babron, M. Bourgey, A. S. Louka, F. Clot, S. Percopo, I. Coto, J. P. Hugot, H. Ascher, L. M. Sollid, L. Greco, and F. Clerget-Darpoux. 2004. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. *Tissue Antigens* 63:562-7.

175. Melanitou, E., P. Fain, and G. S. Eisenbarth. 2003. Genetics of type 1A (immune mediated) diabetes. *J Autoimmun* 21:93-8.

176. Hardin, J. A., L. Donegan, R. C. Woodman, C. Trevenen, and D. G. Gall. 2002. Mucosal inflammation in a genetic model of spontaneous type I diabetes mellitus. *Can J Physiol Pharmacol* 80:1064-70.

177. Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat.Rev.Immunol.* 3:331-341.

178. Maurano, F., G. Mazzarella, D. Luongo, R. Stefanile, R. D'Arienzo, M. Rossi, S. Auricchio, and R. Troncone. 2005. Small intestinal enteropathy in non-obese diabetic mice fed a diet containing wheat. *Diabetologia* 48:931-7.

179. MacFarlane, A. J. Identification and Characterization of the type 1 diabetes-related wheat storage globulin WP5212. Thesis . 2004. Department of Biochemistry, Microbiology and Immunology, Faculty of medicine, University of Ottawa.
Ref Type: Thesis/Dissertation

180. Kitts, P. A. and R. D. Possee. 1993. A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* 14:810-817.

181. Webb, N. R., C. Madoulet, P. F. Tosi, D. R. Broussard, L. Sneed, C. Nicolau, and M. D. Summers. 1989. Cell-surface expression and purification of human CD4 produced in baculovirus-infected insect cells. *Proc.Natl.Acad.Sci.U.S.A* 86:7731-7735.

182. Terpe, K. 2003. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl.Microbiol.Biotechnol.* 60:523-533.

183. Seidler, A. 1994. Introduction of a histidine tail at the N-terminus of a secretory protein expressed in Escherichia coli. *Protein Eng* 7:1277-1280.

184. Barera, G., R. Bonfanti, M. Viscardi, E. Bazzigaluppi, G. Calori, F. Meschi, C. Bianchi, and G. Chiumello. 2002. Occurrence of celiac disease after onset of type 1 diabetes: a 6-year prospective longitudinal study. *Pediatrics* 109:833-838.

185. Barera, G., C. Bianchi, L. Calisti, F. Cerutti, F. Dammacco, E. Frezza, M. T. Illeni, L. Mistura, M. Pocecco, F. Prisco, and . 1991. Screening of diabetic children for coeliac disease with antigliadin antibodies and HLA typing. *Arch.Dis.Child* 66:491-494.

186. Vitoria, J. C., L. Castano, I. Rica, J. R. Bilbao, A. Arrieta, and M. D. Garcia-Masdevall. 1998. Association of insulin-dependent diabetes mellitus and celiac disease: a study based on serologic markers. *J.Pediatr.Gastroenterol.Nutr.* 27:47-52.

187. Ludvigsson, J. F., J. Ludvigsson, A. Ekbom, and S. M. Montgomery. 2006. Celiac disease and risk of subsequent type 1 diabetes: a general population cohort study of children and adolescents. *Diabetes Care* 29:2483-2488.

188. Gottschall E. *Breaking the vicious cycle: Intestinal health through diet.* Baltimore:Kirkton Press Ltd..

189. Sollid, L. M. 2002. Coeliac disease: dissecting a complex inflammatory disorder. *Nat.Rev.Immunol.* 2:647-655.

190. Bonifacio, E., A. G. Ziegler, M. Hummel, J. Dittler, V. Lampasona, M. R. Pastore, and E. Bosi. 1998. Gluten: is it also a determinant of islet autoimmunity? *Diabetes Metab Rev* 14:258-9.

191. Troncone, R., A. Franzese, G. Mazzarella, F. Paparo, R. Auricchio, I. Coto, M. Mayer, and L. Greco. 2003. Gluten sensitivity in a subset of children with insulin dependent diabetes mellitus. *Am J Gastroenterol* 98:590-5.

192. Rewers M, Mojibian M, Norris J, MacFann K, and Scott F.W. Glb1 homologue antibodies and celiac disease. XII International Celiac Disease Symposium, XII International Celiac Disease Symposium, New York City, Nov 9-11. 2006.
Ref Type: Conference Proceeding

193. Charlton, B. and K. J. Lafferty. 1995. The Th1/Th2 balance in autoimmunity. *Curr.Opin.Immunol.* 7:793-798.

194. Kidd, P. 2003. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern.Med.Rev.* 8:223-246.

195. Charles A.Janeway, Paul Travers, and Mark Walport & Mark Shlomchik. *Immunobiology (The immune system in health and disease)*, Vol. 6th Edition.

196. Saalman, R., U. I. Dahlgren, S. P. Fallstrom, L. A. Hanson, S. Ahlstedt, and A. E. Wold. 2001. IgG subclass profile of serum antigliadin antibodies and antibody-dependent cell-mediated cytotoxicity in young children with coeliac disease. *Scand.J.Immunol.* 53:92-98.

197. Saalman, R., U. I. Dahlgren, S. P. Fallstrom, L. A. Hanson, S. Ahlstedt, and A. E. Wold. 2003. Avidity progression of dietary antibodies in healthy and coeliac children. *Clin.Exp.Immunol.* 134:328-334.

198. Fasano, A., T. Not, W. Wang, S. Uzzau, I. Berti, A. Tommasini, and S. E. Goldblum. 2000. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* 355:1518-1519.

199. van Wijk, F. and L. Knippels. 2007. Initiating mechanisms of food allergy: Oral tolerance versus allergic sensitization. *Biomed.Pharmacother.* 61:8-20.

200. Strobel, S. and A. M. Mowat. 1998. Immune responses to dietary antigens: oral tolerance. *Immunol.Today* 19:173-181.

201. Fasano, A. and T. Shea-Donohue. 2005. Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat.Clin.Pract.Gastroenterol.Hepatol.* 2:416-422.

202. Smecuol, E., E. Sugai, S. Niveloni, H. Vazquez, S. Pedreira, R. Mazure, M. L. Moreno, M. Label, E. Maurino, A. Fasano, J. Meddings, and J. C. Bai. 2005. Permeability, zonulin production, and enteropathy in dermatitis herpetiformis. *Clin.Gastroenterol.Hepatol.* 3:335-341.

203. Battais, F., F. Pineau, Y. Popineau, C. Aparicio, G. Kanny, L. Guerin, D. A. Moneret-Vautrin, and S. Denery-Papini. 2003. Food allergy to wheat: identification of immunogloglin E and immunoglobulin G-binding proteins with sequential extracts and purified proteins from wheat flour. *Clin.Exp.Allergy* 33:962-970.

204. Daengsuwan, T., K. Palosuo, S. Phankingthongkum, N. Visitsunthorn, O. Jirapongsananuruk, H. Alenius, P. Vichyanond, and T. Reunala. 2005. IgE antibodies to omega-5 gliadin in children with wheat-induced anaphylaxis. *Allergy* 60:506-509.

205. Courtois, P., G. Nsimba, H. Jijakli, A. Sener, F. W. Scott, and W. J. Malaisse. 2005. Gut permeability and intestinal mucins, invertase, and peroxidase in control and diabetes-prone BB rats fed either a protective or a diabetogenic diet. *Dig.Dis.Sci.* 50:266-275.

206. Scott, F. W., P. Rowsell, G. S. Wang, K. Burghardt, H. Kolb, and S. Flohe. 2002. Oral exposure to diabetes-promoting food or immunomodulators in neonates alters gut cytokines and diabetes. *Diabetes* 51:73-8.

207. Mojibian M, Lefebvre D.E, Chakir H, and Scott F.W. T-cell reactivity to wheat proteins in human type 1 diabetes (abstract). Diabetes 54:A79 Diabetes 54:A79. 2005. Ref Type: Abstract

208. Simpson M.D, Mojibian M, Barriga K, Fasano A, Scott F.W, Rewers M, and Norris J.M. Correlation between serum zonulin and antibodies to a wheat storage globulin homologue of Glb1, among childeren with islet autoimmunity (IA). 67th Scientific Sessions - Annual Meeting of American Diabetes association 67th Scientific Sessions- Annual Meeting of American Diabetes Association. 2007. Ref Type: Abstract

209. Mojibian M, Strom A, MacFarlane A.J, and Scott F.W. Immune reactivity to a homologue of wheat storage globulin,Glb1 in human type 1 diabetes. Experimental Biology Annual Meeting; April28-May2 Experimental Biology Annual Meeting; April28-May2. 2007. Ref Type: Abstract

210. Whalen, B. J., L. C. Love, J. P. Mordes, A. A. Rossini, and D. L. Greiner. 1999. Intravital dye-labeled diabetogenic rat T cells retain dye, home to the pancreas, and induce diabetes. *Transplant.Proc.* 31:1611-1614.

211. Albert, L. J. and R. D. Inman. 1999. Molecular mimicry and autoimmunity. *N.Engl.J.Med.* 341:2068-2074.

212. Christen, U. and M. G. von Herrath. 2004. Induction, acceleration or prevention of autoimmunity by molecular mimicry. *Mol.Immunol.* 40:1113-1120.

213. Honeyman, M. C., N. L. Stone, and L. C. Harrison. 1998. T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents. *Mol.Med.* 4:231-239.

214. Alaedini, A., H. Okamoto, C. Briani, K. Wollenberg, H. A. Shill, K. O. Bushara, H. W. Sander, P. H. Green, M. Hallett, and N. Latov. 2007. Immune cross-reactivity in celiac disease: anti-gliadin antibodies bind to neuronal synapsin I. *J.Immunol.* 178:6590-6595.

215. Wraith, D. C., M. Goldman, and P. H. Lambert. 2003. Vaccination and autoimmune disease: what is the evidence? *Lancet* 362:1659-1666.

216. Westerholm-Ormio, M., O. Vaarala, P. Pihkala, J. Ilonen, and E. Savilahti. 2003. Immunologic activity in the small intestinal mucosa of pediatric patients with type 1 diabetes. *Diabetes* 52:2287-95.

217. Cronin, C. C. and F. Shanahan. 1997. Insulin-dependent diabetes mellitus and coeliac disease. *Lancet* 349:1096-1097.

218. Ventura, A., G. Magazzu, and L. Greco. 1999. Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. SIGEP Study Group for Autoimmune Disorders in Celiac Disease. *Gastroenterology* 117:297-303.

STATEMENT OF CONTRIBUTION OF COLLABORATORS

Dr. Amanda MacFarlane, Dr. David Lefebvre, Dr Karolina Burghardt and Dr. Habiba Chakir
Former graduate students and postdoctoral fellow in the Scott laboratory
Ottawa Health Research Institute
Ottawa, Ontario, Canada

- Dr. MacFarlane contributed to case report manuscript and construction of the pET17-His-Glb1 which I used for isolation of the His-Glb1 DNA fragment
- Dr. Lefebvre and Dr. Chakir contributed to the initiation and generation of some preliminary data for the study of young adult patients
- Dr. Burghardt provided original extracts of wheat protein antigens

Dr. Alexander Strom
Postdoctoral fellow in the Scott laboratory
Ottawa Health Research Institute
Ottawa, Ontario, Canada

- Dr Strom shared his expertise and assisted with the cloning of Glb1, expression of the protein in SF21 cells and purification

Ms. Jennifer Crookshank and Ms. Brigitte Sonier
Study coordinators in the Scott laboratory
Ottawa Health Research Institute
Ottawa, Ontario, Canada

- Ms. Crookshank and Ms. Sonier recruited patients and controls, coordinated the study and assisted with separation of serum

Dr. Claire Touchie, Dr. Jacob Karsh, Dr. Erin Keely, Dr. Rene Wong and Dr. Janine Malcolm
Department of Medicine
The Ottawa Hospital
Ottawa, Ontario, Canada

- Drs. Touchie and Karsh recruited the case study patient and assisted with the manuscript and the other physicians recruited patients for the human study

Dr. Sarah Lawrence[1] and Dr. David Mack[2]
[1]Division of Endocrinology
[2]Division of Gastroenterology
Children's Hospital of Eastern Ontario
Ottawa, Ontario, Canada

- Dr. Lawrence and Dr. Mack assisted with patient recruitment

Dr. Marian Rewers[1] and Dr. Jill Norris[2]
[1] Barbara Davis Center for Childhood Diabetes
[2] Department of Preventive Medicine and Biometrics
University of Colorado, School of Medicine
Denver, Colorado

- Dr. Rewers and Dr. Norris provided samples from the DAISY and CEDAR prospective studies and performed some of the statistical analyses

COPYRIGHT PERMISSION

Christine Taylor
Associate
Rights and Permissions
Publications
703-549-1500 ext. 1655 - Tel
703-683-2890 - Fax
permissions@diabetes.org

June 19, 2007

Mojibian Majid
Ottawa Health Research Institute
501 Smyth Road
Ottawa, Ontario
K1H 8L6

Permission Request Number: ***CT052507-OHRI***

Dear Mojibian Majid:

We are pleased to grant permission to you reproduce the following article:

✓ **Immune Reactivity to a Glb1 Homologue in a Highly Wheat-Sensitive Patient With Type 1 Diabetes and Celiac Disease**

From: **Diabetes Care®, Vol. 29, 2006; 1108-1110**
For use in: **Thesis**

This permission is a one-time, **non-exclusive** grant for English language use, for a period of **twelve (12) months**, beginning from the date of this letter.

This permission is subject to the following conditions:

1. Permission is granted for print usage only, no permission is granted for electronic use.
2. Each copy containing our material that you reproduce or distribute must bear the following copyright notice:

> **"Copyright © 2006 American Diabetes Association**
> **From Diabetes Care®, Vol. 29, 2006; 1108-1110**
> **Reprinted with permission from *The American Diabetes Association.*"**

Sincerely,

Christine N. Taylor

Unless specifically noted here, the permission granted does not include the use of the American Diabetes Association logo or the cover logo of the journal(s). Permission to reproduce material does not permit the above-named entity or the company sponsoring the resulting product to act as an agent of the American Diabetes Association or of the journals(s).

CURRICULUM VITAE

Majid Mojibian

EDUCATION

2001-2007 University of Ottawa, Ottawa, ON
Doctorate in Philosophy Microbiology and Immunology

- Thesis: Immune response to potential diabetes-related dietary antigens, including Glb1 in patients with type 1 diabetes

1988-1993 Shahid Beheshti University, Tehran, Iran
Doctorate in Clinical Laboratory Science

- Thesis: Diagnosis of TB pleural effusion through measuring ADA and lysozyme

1983-1986 Iran University, Tehran, Iran
B.Sc. Medical Laboratory Science

WORK EXPERIENCE

2004-present Ottawa Health Research Institute
Ph.D. candidate, Immune response to potential diabetes-related dietary antigens, including Glb1 in patients with type 1 diabetes

2001-2004 University of Ottawa
[†]*Ph.D. candidate,* Human immune response to *Leishmania* recombinant antigens

2002 Faculty of Science, University of Ottawa
Teaching Assistant, Biochemistry Laboratory, BCH 2336

2000-2001 University of Ottawa
Research Assistant, Immunology laboratory

1998-1999 Ahang Medical Diagnostic Laboratory, Tehran, Iran
Lab Director and supervisor

[†] My First supervisor left the University in 2004 to take up a position in Victoria, B.C.

1996-1999 Chizar Medical Diagnostic Laboratory, Tehran, Iran
Lab Supervisor

1995-1996 Ghiyasi Charity Hospital, Tehran, Iran
Lab supervisor, Established the clinical laboratory,

1994-1995 Medical Laboratory of Jihad-daneshghahi, Yazd, Iran
Director and supervisor, Established the clinical laboratory,

1993-1995 Faculty of Medicine and Para medicine, University of Yazd
Lecturer, Virology, theory and practical, to clinical laboratory technologist

1993-1995 Central Clinical Laboratory of Yazd University, Yazd, Iran
Lab Supervisor, Director of Training

1989-1993 Clinical Laboratory, Noor-Afshar Hospital, Tehran, Iran
Lab Technologist

1989-1989 Clinical Laboratory, Mehrad General Hospital, Tehran, Iran
Lab Technologist

1986-1988 Clinical Laboratory, Health Center, Iranshahr and Andimeshk, Iran
Lab Technologist

PUBLICATIONS

Chakir H; Campos-Neto A, **Mojibian M** and Webb JR. Genetically Resistant Mice Lacking IL-12Rβ2 gene are susceptible to *Leishmania major* infection and develop a specific Th2 Immune Response. (2003). Microbes and Infection 5 (4):241-9.

Mojibian M, Chakir H, MacFarlane AJ, Lefebvre DE, Webb JR, Touchie C, Karsh J, Crookshank JA, Scott FW. Immune reactivity to a Glb1 homologue in a highly wheat-sensitive patient with type 1 diabetes and celiac disease. (2006) Diabetes Care. May;29(5):1108-10

Azizi A, Ghorbani M, **Mojibian M**, Diaz-Mitoma F. Synergistic effect of combined HIV/HCV immunogens: A combined HIV-1/HCV candidate vaccine induces a higher level of CD8+ T cell immune responses in HLA-A2.1 mice. Curr HIV Res. 2007 Apr ;5 (2):211-9

MANUSCRIPTS IN PREPARATION

Mojibian M, Chakir H, Lefebvre DE, Crookshank J, Scott FW. Increased T cell immune response to wheat proteins in patients with type 1 diabetes.

Mojibian M, Strom A, MacFarlane A, Kolb H, Walz M, Sonier B, Scott FW. Immune response to a wheat storage globulin-like protein, Glb1, in patients with type 1 diabetes.

Simpson M.D†, **Mojibian M**†, Strom A, Barriga K, Fasano A, Scott F.W, Rewers M, Norris J.M. Glb1 homologue antibodies and correlation with serum zonulin among children with islet autoimmunity (IA).

Strom A, Slatculescu C, Sonier B, **Mojibian M**, Wang Gen-Sheng, Scott FW . Antibody reaction against a 58 kDa insulinoma protein, PRPH, is not exclusively related to type 1 diabetes.

Patrick C †, Wang GS †, Lefebvre DE, Crookshank JA , **Mojibian M,** Scott FW. Alterations in gut immune status parallel diet-related autoimmune diabetes in germ-free diabetes-prone rats.

ABSTRACTS / PRESENTATION AT SCIENTIFIC MEETINGS

2007 12th EASD/JDRF Oxford Workshop to be held at Keble College, Oxford, UK
Oral Presentation: Evidence for dietary modification of T1D (Immune reactivity to wheat proteins in human type 1 diabetes). Scott F.W and **Mojibian M.**

2007 American Diabetes Association 67th Scientific Sessions, Chicago, IL
Abstract: correlation between serum zonulin and antibodies to a wheat storage globulin homologue of Glb1, among childeren with islet autoimmunity (IA) Simpson M.D, **Mojibian M,** Barriga K, Fasano A, Scott F.W, Rewers M, Norris J.M.

2007 Experimental Biology 2007, Washington, DC
Poster Presentation: Immune reactivity to a homologue of wheat storage globulin, Glb1 in human type 1 diabetes. **Mojibian M,** Strom A, MacFarlane A.J, Scott F.W.

2006 XII International Celiac Disease Symposium, New York City, NY
Abstract: Glb1 homologue antibodies and celiac disease. Rewers M, **Mojibian M,** Norris J, MacFann K, Scott F.W.

2006 First Nordic Meeting on Genetics and Pathogenesis of Immunological Diseases, Helsinki, Finland
Poster Presentation: Wheat Protein Immune Reactivity in Human Type 1 Diabetes Linked to HLA-DR4. **Mojibian M**, Chakir H, Lefebvre DE, Scott FW.

2005 American Diabetes Association 65th Scientific Meetings
Oral Presentation: T cell reactivity to wheat proteins in human type 1 diabetes. **Mojibian M**, Lefebvre DE, Chakir H, Scott FW. 2005. Diabetes 54:A79 Supp.1.

2005 Ottawa Health Research Institute Research Day Diabetes and Obesity Symposium, Ottawa, ON
Oral Presentation: Abnormal T cell reactivity to wheat proteins in patients with type 1 diabetes is linked with the HLA-DR4 risk gene. **Mojibian M**, Lefebvre DE, Chakir H, Scott FW.

2004 The BB Rat Turns Thirty: Lessons and Future Directions – International Symposium Meeting, Ottawa, ON
Poster Presentation: Increased T cell immune response to wheat proteins in patients with type 1 diabetes. **Mojibian M,** Lefebvre DE, Chakir H, Scott FW.

2004 Ontario HIV Treatment Network (OHTN), Toronto, ON
Abstract: Immunogenicity Of Single and Combined HIV-1/HCV Candidate Vaccines In a HLA-A2.1 Murine Model. Azizi A, Ghorbani M, **Mojibian M,** Montefiori D, Diaz- Mitoma F.

2002 Woods Hole ImmunoParasitology meeting, (WHIP) -Woods Hole, Massachusetts
Poster Presentation: Human Immune responses to *Leishmania* recombinant antigens. **Mojibian M,** and Webb J.

1994 5th International Biochemistry Congress, Tehran, Iran
Oral Presentation: Differential Diagnosis of TB effusion through measuring ADA and Lysozyme. **Mojibian M**, Keyhani M, Hajipoor R, Velayati A, Masjedi M.

PATENT APPLICATION

2004 "Diabetogenic Epitopes" FW Scott, AJ MacFarlane, KM Burghardt, **M Mojibian** USA, Canada and EU

HONOURS AND AWARDS

2005	University of Ottawa *Microbiology and Immunology, Faculty of Graduate and Post-Doctoral Studies Travel Award*
2003-2004	Canadian Blood Services *Graduate Fellowship Award, Renewal ($20,000/year)*
2002	University of Ottawa *Department of Microbiology and Immunology Travel Award*
2002	University of Ottawa *Microbiology and Immunology Graduate student Poster Day* *First Place Ph.D. poster*

2002-2005	University of Ottawa *National Excellence Scholarship ($5235/year)*
2001–2003	Canadian Blood Services *Graduate Fellowship Award ($19,000/year)*
2001-2002	University of Ottawa *Doctoral Admission Scholarship Award ($5235/year)*