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Inflammatory immune reactivity to cereal proteins in type 1 diabetes

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Inflammatory immune reactivity to cereal proteins in type 1 diabetes

David E. Lefebvre

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the PhD degree in biochemistry

Biochemistry, Microbiology and Immunology
Faculty of Science
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-49374-8
Our file Notre référence
ISBN: 978-0-494-49374-8

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Inflammatory immune reactivity to cereal proteins in type 1 diabetes

Background: Type 1 diabetes (T1D) is an autoimmune disease in which a patient's immune system destroys the insulin-secreting beta-cells in the pancreas. The origin of beta-cell-specific leukocytes and the identity of the stimulatory antigens remain unknown. Disease development in individuals with genetic risk is thought to require environmental triggers. Epidemiological data and evidence from animal models suggests that exposure to dietary wheat can enhance diabetes autoimmunity. Some evidence suggests a crucial role of the gut barrier and gut-associated lymphoid tissues (GALT). **Hypothesis:** The gut in T1D susceptible rodents and patients is dysfunctional and characterized by a pro-inflammatory immune microenvironment which is associated with a loss of tolerance to cereal proteins. **Methods:** The inflammatory status of the GALT of diabetes-prone BioBreeding (BBdp) rats was quantified by RT-PCR, ELISA, and immunoblotting for T helper 1 / T helper 2 (Th1/Th2) cytokine markers. T cell reactivity to wheat proteins was quantified by flow cytometric proliferation assay with cells from the GALT of BBdp rats and with peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes. The effect of microbes and diet on diabetes outcome was characterized by feeding trials in germ-free BBdp rats. **Results:** T-bet/GATA-3 transcription factor expression ratio reflected Th1/Th2 immune cell status in the BB rat and provided a snapshot of the *in vivo* inflammatory status of tissues. At an age which pre-dates the development of T1D, the GALT of BBdp rats, fed a diabetes-promoting cereal-based diet, contained an unusually high proportion of pro-inflammatory Th1 cells. These T cells specifically responded to wheat proteins *in vitro*. Similarly, T cell reactivity to wheat proteins was also observed in PBMCs in a subset of patients with T1D. PBMCs from a patient with T1D and celiac disease-associated gut damage revealed strong Th1-biased immune reactivity to wheat protein. Diabetes was promoted by a cereal-based diet in both the presence and absence of microbes in BBdp rats, and a diabetes-promoting effect of microbes was revealed in the absence of cereal proteins. **Conclusions:** At ages which precede diabetes, a pro-inflammatory state in the gut and GALT of BBdp rats is associated with the activation of wheat-specific T cells. An immune response to wheat is also present in PBMCs in a subset of patients with T1D. A low-antigen diet inhibited the development of diabetes in BBdp rats and almost completely prevented diabetes in germ-free BBdp rats. These findings suggest that the gut could play an important role in the development of T1D.

ABSTRACT

Background: Type 1 diabetes (T1D) is an autoimmune disease in which a patient's immune system destroys the insulin-secreting beta-cells in the pancreas. The origin of beta-cell-specific leukocytes and the identity of the stimulatory antigens remain unknown. Disease development in individuals with genetic risk is thought to require environmental triggers. Epidemiological data and evidence from animal models suggests that exposure to dietary wheat can enhance diabetes autoimmunity. Some evidence suggests a crucial role of the gut barrier and gut-associated lymphoid tissues (GALT). **Hypothesis:** The gut in T1D susceptible rodents and patients is dysfunctional and characterized by a pro-inflammatory immune microenvironment which is associated with a loss of tolerance to cereal proteins. **Methods:** The inflammatory status of the GALT of diabetes-prone BioBreeding (BBdp) rats was quantified by RT-PCR, ELISA, and immunoblotting for T helper 1 / T helper 2 (Th1/Th2) cytokine markers. T cell reactivity to wheat proteins was quantified by flow cytometric proliferation assay with cells from the GALT of BBdp rats and with peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes. The effect of microbes and diet on diabetes outcome was characterized by feeding trials in germ-free BBdp rats. **Results:** T-bet/GATA-3 transcription factor expression ratio reflected Th1/Th2 immune cell status in the BB rat and provided a snapshot of the *in vivo* inflammatory status of tissues. At an age which pre-dates the development of T1D, the GALT of BBdp rats, fed a diabetes-promoting cereal-based diet, contained an unusually high proportion of pro-inflammatory Th1 cells. These T cells specifically responded to wheat proteins *in vitro*. Similarly, T cell reactivity to wheat proteins was also observed in PBMCs in a subset of patients with T1D. PBMCs from a patient with T1D and celiac disease-associated gut

damage revealed strong Th1-biased immune reactivity to wheat protein. Diabetes was promoted by a cereal-based diet in both the presence and absence of microbes in BBdp rats, and a diabetes-promoting effect of microbes was revealed in the absence of cereal proteins. **Conclusions:** At ages which precede diabetes, a pro-inflammatory state in the gut and GALT of BBdp rats is associated with the activation of wheat-specific T cells. An immune response to wheat is also present in PBMCs in a subset of patients with T1D. A low-antigen diet inhibited the development of diabetes in BBdp rats and almost completely prevented diabetes in germ-free BBdp rats. These findings suggest that the gut could play an important role in the development of T1D.

To Mom, Dad, Katherine, and Cam for your love and support,

and especially to my wife Jaclyn for your constant loving encouragement.

"When we realize how vast and beautiful the creation is, we are learning about the creator at the same time." (Wisdom of Solomon 13:5, Good News Bible)

ACKNOWLEDGEMENTS

I thank my thesis supervisor, Dr. Fraser Scott, for his commitment and support as my mentor. His leadership, encouragement, and friendship made my graduate studies productive and enjoyable. I would also like to thank my lab mates in Dr. Scott's lab as well as professors and support staff from the Department of Biochemistry, Microbiology and Immunology at the University of Ottawa and from the Ottawa Health Research Institute for their help and encouragement. I acknowledge the collaborators who have made intellectual contributions to this work, namely Drs. Haiping Wang, Habiba Chakir, Majid Mojibian, and Gen-Sheng Wang, as well as Mr. Chris Patrick. I thank the members of my thesis advisory committee; Drs. Illimar Altosaar, Anthony Krantis, Ashok Kumar, and Sylvia Vidal for their guidance. I acknowledge my defence examiners; Drs. Philippe Poussier, Lionel Filion, Paul MacPherson, and Alain Stintzi for their helpful suggestions. Many thanks for the technical assistance provided by Ms. Heidi Gruber, Ms. Amy Shih, Ms. Jennifer Crookshank, Mr. Ahmed Zafer, Mr. Elliott Faller, Ms. Brigitte Sonier, Mr. Jocelyn Souligny and Mr. Dominique Patry. I express my appreciation to the volunteers who participated in these studies. Funding for this work was provided to Dr. Fraser Scott by the Juvenile Diabetes Research Foundation, the Canadian Institutes of Health Research, the Canadian Diabetes Association, the Ontario Research and Development Challenge Fund, the Canada Foundation for Innovation, and Health Canada. I thank the Ontario Ministry of Training and Colleges and Universities for funding my Ontario Graduate Scholarships, and the University of Ottawa for awarding scholarships and travel grants.

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LIST OF ABBREVIATIONS

ANOVA, analysis of variance

APC, antigen presenting cell

BBc, BioBreeding control rat

BBdp, diabetes-prone BioBreeding

BSA, bovine serum albumin

Cbl-b, Casitas B cell lineage lymphoma-b gene

CD, celiac disease

CER, cereal

CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester

DAISY, Diabetes Autoimmunity in the Young study

DIPP, Diabetes Prediction and Prevention study

ELISA, enzyme-linked immunosorbent assay

FBS, fetal bovine serum

FITC, fluorescein isothiocyanate

GAD, glutamic acid decarboxylase-65

GALT, gut-associated lymphoid tissue

GATA-3, GATA-binding protein 3

GF, germ-free

Glb1, homologue of the wheat storage globulin-1 protein

HC, diabetes-retardant AIN-93G hydrolyzed casein-based diet

HLA, human leukocyte antigen

IA-2, insulinoma-associated autoantigen-2

IDDM, insulin-dependent diabetes mellitus

IEL, intraepithelial lymphocyte

IFN, interferon

IL, interleukin

KDP, Komeda diabetes-prone

LSD, least significant difference post-hoc test

mAb, monoclonal antibody

MHC, major histocompatibility complex

MLN, mesenteric lymph nodes

MOPS, 4-morpholinepropanesulfonic acid

NK, natural killer

NOD, non-obese diabetic

NTP-2000, diabetes-promoting, cereal-based diet

OVA, chicken egg white ovalbumin protein

PBMC, peripheral blood mononuclear cell

PE, phycoerythrin

PHA, phytohemagglutinin

PMA, phorbol 12-myristate 13-acetate

PP, Peyer's patch

RFLP, restriction fragment length polymorphism

RT-PCR, reverse transcription-polymerase chain reaction

SD, standard deviation

SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SPF, specific pathogen-free

SPRD, spectra red

T1D, type 1 diabetes

T-bet, T-box expressed in T cells

TCR, T-cell receptor

TGF, transforming growth factor

Th1, T helper 1

Th2, T helper 2

TLR, Toll-like receptor

TRIGR, Trial to Reduce IDDM in the Genetically at Risk study

WP, wheat proteins

INTRODUCTION

Autoimmune Diseases

Autoimmune diseases, such as type 1 diabetes (T1D), involve an immune response directed at self antigens (1). Genetic predisposition is a major risk factor for many autoimmune diseases and environmental factors are often believed to trigger onset (2). The incidence of autoimmune diseases is increasing (3). It will be important to elucidate the identity of environmental triggers and to determine how they act in order to develop strategies to treat and prevent these diseases.

The Immune System

The mammalian immune system evolved to recognize and eliminate foreign pathogens and toxins while maintaining tolerance to self tissues (4). The innate immune response is an immediate, non-specific response to microbes, which is mediated primarily by phagocytic cells such as macrophages and dendritic cells. The adaptive immune response involves the development of long-term immunity to a specific pathogen. This response is mediated by lymphocytes including B cells and T cells, which are activated during the infection and maintained following its clearance.

An adaptive immune response involves the specific recognition of antigens, usually proteins, produced by an invading pathogen. B cells are activated when cognate antigen is recognized by the cell-surface B cell receptor. Activated B cells differentiate into plasma cells and secrete antibodies. The T cell receptor (TCR) on the surface of T cells recognizes antigens presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs), including dendritic cells, macrophages, and B cells. Upon

recognition of their cognate antigen, T cells are activated, proliferate, secrete intercellular signaling proteins called cytokines, and mediate adaptive immunity (5).

By the classical definition, CD8 cytotoxic T cells recognize intracellular antigens presented on MHC class I molecules by all nucleated cells of the body and can lyse infected cells (4). CD8 cytotoxic T cells can kill infected cells. CD4 T cells are activated by exogenous antigens presented on MHC class II molecules by APCs. Phagocytosed particles such as bacterial components as well as dietary antigens can be presented this way. CD4 T cells secrete cytokines which direct the nature of the immune response. T helper 1 (Th1) cytokines induce an inflammatory, cell-mediated immune response while T helper 2 (Th2) cytokines promote antibody-mediated responses.

The fundamental initial step in the development of CD4 T cell response and secretion of Th1 or Th2 cytokines is the activation of a naïve T cell by TCR recognition of antigen presented on MHC class II molecules. Depending on the nature of the immune challenge, activated naïve cells enact genetic programs that result in differentiation towards either the Th1 or Th2 lineages. Several factors determine the differentiation of activated T cells including antigen concentration, phenotype of APCs, ligation of co-stimulatory molecules, chromatin structure, and most importantly the cytokines present in the cell's microenvironment (6). Cytokines are glycoproteins which regulate immune function by binding to receptors on and transmitting signals to immune cells in an autocrine and/or paracrine manner (5). They induce further cytokine secretion and promote effector responses to clear infection.

The differentiation of naïve CD4 T cells to Th1 cells is regulated by the transcription factor T box expressed in T-cells (T-bet) and differentiation to Th2 cells is regulated by GATA-binding protein-3 (GATA-3). The fate of a developing CD4 T cell may be determined by the relative predominance of T-bet and GATA-3. T-bet, which is expressed in $CD4^+$ Th1, $CD8^+$ T cytotoxic 1 and natural killer (NK) cells but not Th2 cells (Table 1-1), induces the secretion of the Th1 cytokine interferon- γ (IFN-γ) by STAT-4 signaling (7). GATA-3, which is expressed in several cell types including Th2 but not Th1 cells (Table 1-1), induces secretion of the Th2 cytokines Interleukin-4 (IL-4) and IL-5 which in turn induce the secretion of IL-10 and IL-13 (8).

Developing T cells which are specific for self proteins are usually deleted or become anergic during selection in the thymus. However, even in healthy individuals, autoreactive T cells occasionally escape selection. These autoreactive cells are normally controlled by peripheral tolerance mechanisms including regulatory T cells. Failure to properly control these autoreactive cells can result in the development of an autoimmune disease (1).

Table 1-1. Cells which express T-bet, GATA-3, IFN-γ, and IL-4, and cytokines which are classified as having Th1 or Th2 function

T-bet-EXPRESSING CELLS	REF.	GATA-3-EXPRESSING CELLS	REF
CD4 Th1 cells	(6, 7)	Thymocytes	(9)
CD8 T cytotoxic 1 cells	(10)	Naïve CD4 T cells	(8, 9)
NK cells	(10, 11)	CD4 Th2 cells	(6, 8)
NKT cells	(11)	Mast cells	(12)
γδ T cells	(13)	Eosinophils	(12)
Monocytes	(14)	Basophils	(12)
Macrophages	(11, 14)		
Dendritic cells	(11, 14)		
B cells	(11, 14)		

IFN-γ-EXPRESSING CELLS		IL-4-EXPRESSING CELLS	
CD4 Th1 cells	(15)	CD4 Th2 cells	(16, 17)
CD8 T cytotoxic 1 cells	(6, 17)	CD8 T cytotoxic 2 cells	
Macrophages	(16)	Mast cells	(6, 18)
NK cells	(6, 16)	Basophils	(6)
γδ T cells	(19)	Eosinophils	(6)
Dendritic cells	(20, 21)	B cells	(6)
B cells	(22)	$NK1.1^+$ $CD4^+$ T cells	(6, 23)
		γδ T cells	(13)

Th1 CYTOKINES		Th2 CYTOKINES	
IFN-γ	(6, 15)	IL-4	(6, 17, 24)
TNF-β	(6)	IL-5	(6, 17, 24)
IFN-α (in human)	(6)	IL-6	(24)
IL-12	(6, 11)	IL-9	(6, 24)
IL-18	(25)	IL-10	(6, 24)
IL-27	(11)	IL-13	(6, 24)
LT	(6)	IL-18	(25)
		GM-CSF	(24)
		Macrophage-derived factor	(24)

Type 1 Diabetes

Type 1 diabetes mellitus (T1D) is a spontaneous autoimmune disease that occurs in children and young adults (2). The prevalence is approximately 0.5 % in developed countries. The pathogenesis of T1D involves chronic, inflammatory leukocyte-mediated autoimmune destruction of insulin-secreting beta-cells in the pancreas. Insulin is an endocrine hormone which permits cellular uptake and metabolism of blood glucose. Patients with T1D lack insulin and must monitor blood glucose and inject insulin to control their glucose level. If blood glucose is not maintained within a physiological margin, a difficult task for many patients, serious secondary complications can occur. Pre-diabetic individuals begin life with normal pancreatic beta-cell mass and insulin levels. When individuals undergoing diabetes autoimmunity lose approximately 90 % of the beta-cell mass in the pancreas, they lack sufficient insulin to maintain normoglycemia and diabetes is diagnosed. Loss of circulating insulin results not only in dysregulated glucose, but also in abnormal protein and lipid homeostasis. The cause of diabetes autoimmunity remains unknown and despite many years of research there is still no cure (2).

An individual's risk of developing T1D is associated with more than 20 genetic loci (26). The greatest genetic risk comes from alleles in the MHC region, called human leukocyte antigen (HLA) in humans. Most T1D patients carry the HLA alleles DR3 in combination with DQ8, or DR4 in combination with DQ2. The region encoding these genes is referred to as *IDDM1* (27). Other risk genes of interest include *IDDM2* corresponding to a region located upstream of the insulin gene, *IDDM12* which encodes CTLA-4, PTPN1 which encodes Protein Tyrosine phosphatase 1B, *T-bet* (28), and *toll-like receptor 2* (29).

The autoimmune attack which destroys pancreatic beta-cells is a chronic process. This inflammatory autoimmunity is mediated by the infiltration of several immune cell types into the islets, a process termed insulitis. Macrophages, T cells, and B cells are among the cells which have been detected in the islets of patients newly diagnosed with diabetes (30), and the T cells can have an inflammatory Th1 phenotype (31, 32). Beta-cell-specific autoantibodies can be present and are likely markers of beta-cell destruction. These include antibodies to glutamic acid decarboxylase-65 (GAD), insulinoma-associated autoantigen-2 (IA-2), and pro-insulin (33).

Animal Models Of Spontaneous T1D

Animal models of T1D are useful for the identification of candidate modifiers and pathways involved in the early pre-diabetic phase (34, 35). The major animal models of spontaneous autoimmune T1D are the diabetes-prone BioBreeding (BBdp) rat and nonobese diabetic (NOD) mouse. Komeda diabetes-prone (KDP) rats are a less studied model of spontaneous T1D.

BB rats (35) develop progressive insulitis beginning around adolescence at 50 days that results in diabetes development between 65 and 130 days. The symptoms include loss of circulating insulin, hyperglycemia, failure to gain weight, glucosuria and ketonuria. A T cell deficiency, termed lymphopenia, arises in these animals due to a defect in the GiMap5 gene (36, 37). This immune defect is characterized by reduced CD4 T cells, and greatly diminished CD8 T cells and regulatory T cells (35). The decreased number of regulatory T cells is unable to prevent autoimmunity, allowing the activation of CD4 T cells and a few CD8 T cells, both with a Th1 phenotype, that participate in autoimmune beta-cell destruction (38-41). The earliest cells detected in the islets are macrophages and dendritic cells followed

by CD4 and CD8 T cells, B cells, and natural killer (NK) cells (35). The presence of both CD4 and CD8 T cells is required for diabetes development in this model (42, 43).

Similar to humans, diabetes in BB rats requires multiple risk genes including the MHC class II region (*Iddm1*), the immune-associated nucleotide gene (*Ian4/5 / GiMap5 / Iddm2*), which causes lymphopenia, two susceptibility loci (*Iddm4* and *iddm5*), and polymorphism of a resistance locus (*iddm3*; 35-37, 44).

The NOD mouse develops autoimmune T1D around 80 to 100 days (45). Unlike the case in humans, females have a much greater likelihood of developing diabetes. Mononuclear cell infiltration at the islet border can persist for many weeks and develops into a T cell hyperplasia before the infiltration of autoreactive T cells which induce islet damage. Like the BB rat, diabetes risk in the NOD mouse is associated with multiple risk genes. (34, 45).

Only a fraction of humans with diabetes risk genes develop diabetes (46). Thus it has been proposed that environmental promoting or retardant agents can modify diabetes pathogenesis in individuals with these genes.

Evidence for Environmental Influences on Diabetes Expression

T1D incidence has increased over the past several decades in spite of a decreased frequency of diabetes risk genes in the general population (3, 47). These changes likely involve factors in the environment. The concordance of T1D among monozygotic twins is less than 40 % and is 6 % among siblings (46). This low concordance, in twins who share diabetes risk genes, is consistent with an interaction among risk genes and environmental stimuli.

There is evidence that different environmental factors such as chemicals, bacteria, viruses, and diet could promote diabetes in various subsets of individuals. Several prospective studies have been conducted to identify these factors in humans. These include the Trial to Reduce IDDM in the Genetically at Risk (TRIGR; 48), the Diabetes Prediction and Prevention (DIPP) study (49), the Diabetes Autoimmunity in the Young (DAISY) study (50), and the BABYDIAB study (51). The results of these studies are described later.

Putative Environmental Factors

It has been proposed that viruses can play a role in human T1D(52). Mumps, rubella, cytomegalovirus, retroviruses, and enteroviruses, such as coxsackie, have been implicated (53). However, the DAISY (50) and BABYDIAB (51) prospective studies, and a review of case-control studies (54), all showed no evidence to support a role for coxsackie B in diabetes autoimmunity. Diabetes-prone rodents maintained in specific pathogen-free (SPF) conditions develop diabetes at similar or increased rates compared with those infected with pathogenic viruses (55). This suggests that pathogens are not a required factor for diabetes development. Studies in which dietary carbohydrate, fat, fiber, protein, micronutrients, and caloric intake were examined identified the dietary protein source as the major diabetes modifying dietary component (56). I am the first author of an article in the Annual Review of Nutrition which reviews this topic (2).

Modification of T1D by Dietary Protein Source

Several diabetes-retardant amino acid sources, such as hydrolyzed casein (HC), have been identified in BB rats (56, 57). Diets containing this amino acid source consistently inhibit development of diabetes in BB rats and NOD mice (56, 57). The hydrolysate contains few residual short peptides (most are less than 1 kDa) and is hypoallergenic.

Protection could be due either to the presence of residual diabetes-retardant factors or to the absence of diabetes-promoting components. Various dietary protein sources can promote T1D, including wheat, milk and soy (56, 58). These have been identified by feeding defined semi-purified diets to BB rats and NOD mice (56, 57, 59-63).

A correlation has been demonstrated between early exposure to cow milk and the risk of developing T1D in humans, BB rats, and NOD mice (58, 64, 65). However some studies did not find any relationship (66, 67). The TRIGR dietary intervention pilot study, in which whole dietary proteins were avoided in high-risk neonates (48), provided demonstration of a dietary effect on diabetes autoimmunity in humans. High-risk infants fed a formula containing hydrolyzed casein had a reduced risk of developing islet autoantibodies compared with infants fed a formula containing whole milk (48). Soy proteins present in crude soy preparations (but less so in purified preparations) have been associated with moderate diabetes incidence in NOD mice (68) and BB rats (69). The most potent diabetes-promoting dietary agent in BB rats was wheat gluten protein (57).

Wheat flour contains gluten, which is a mixture of several glutenin and gliadin storage proteins from the wheat endosperm. Albumin and globulin proteins are also present in wheat gluten preparations (70). When BB rats were fed a mainly wheat-based NTP-2000 cereal diet, 65 % of animals developed diabetes (57). Incidence was significantly reduced to 18 % in rats fed an isocaloric, isonitrogenous diet in which hydrolyzed casein was the sole protein source. A defined diet containing wheat gluten as the sole protein source resulted in a 50 % diabetes incidence, indicating that gluten is a major diabetes-promoting component of the NTP-2000 diet. Similar to findings in BB rats, mainly cereal-based diets have also been

reported to be diabetes-promoting in NOD mice compared with gluten-free or hydrolyzed casein diets, although the link with wheat is less clear in NOD mice (60, 68, 71-74).

There is some evidence indicating a role of wheat proteins in human diabetes autoimmunity. The BABYDIAB and DAISY studies reported that exposure to gluten-containing food supplements before 3 months was associated with the development of islet autoantibodies in children with genetic risk of developing type 1 diabetes (75, 76). The DAISY study also indicated increased risk from first gluten exposure after 7 months. In another study, insulin production was improved when newly diagnosed T1D patients consumed a gluten-free diet for 6 months (77). The ongoing BABYDIET study aims to determine if the age of gluten introduction modifies T1D development in susceptible children (78).

Thus, there is considerable evidence that exposure to cereal-based diets promotes the autoimmune destruction of pancreatic beta-cells while a diet which lacks wheat proteins attenuates autoimmunity. However, the mechanism by which diet modifies this process remains unknown. As well, the origin of beta-cell specific T cells and the identity of the stimulatory antigens remain unknown.

Gut Defects and Type 1 Diabetes

The gut immune system responds to microbes and also senses food antigens. The gut mucosal layer is separated from the contents of the lumen by a single epithelial cell layer covered with secreted mucin (79). Several putative diabetes-promoting factors, including dietary proteins and enteroviruses, are first encountered in the gastrointestinal tract.

Increased gut permeability was observed in newly diagnosed T1D patients (80-83). Similar findings have been reported in the pre-insulitis period in BB rats (84, 85).

Paracellular permeability between gut epithelial cells is controlled by tight junction complexes which form a continuous belt around the circumference of the cell. This barrier can selectively allow the passive diffusion of fluid, ions, small macromolecules and leukocytes. Gut permeability also occurs via the trans-cellular route. The increased gut permeability in diabetic individuals appears to occur mainly via the paracellular route and could be related to changes in tight junction complexes (86, 87).

Gut permeability has been directly linked with the development of diabetes in BB rats (87). Zonulin is an endogenous protein that is secreted into the gut lumen and opens tight junctions, thereby increasing gut leakiness. Zonulin levels were significantly increased in the BB rat intestine during the progression of diabetes autoimmunity (87). Decreasing gut permeability by administration of an inhibitor of the zonulin receptor significantly reduced diabetes (87). Similarly, a hydrolyzed casein diet, which decreased gut permeability, also reduced diabetes incidence (85, 88). This suggests that this diet could modify the gut barrier. The close link between gut permeability and the development of diabetes suggests that agents in the gut lumen affect these processes. One explanation is that increased gut permeability is associated with dysregulated glucoregulation, as reflected by insulin:glucose ratio. This could have direct effects on islet function, phenotype and health (88). Another explanation, as yet unconfirmed, is that the dysfunctional gut barrier of some individuals prone to develop T1D facilitates the passage of unusually large amounts of dietary antigens through the mucosa, compromising tolerance to these antigens.

The gut of pre-diabetic BB rats and NOD mice, and of some patients with T1D, shows signs of mild inflammation (73, 89, 90). As early as 30 d, the BB rat displays sporadic enteropathy including flattened villi, crypt hypertrophy and hyperplasia, and

increased T cells in the lamina propria (88, 89, 91). Cell proliferation and metabolic activity were increased in the gut-associated mesenteric lymph nodes (MLN; 92). Gut inflammation in BB rats is marked by changes such as increased peroxidase and a reduction of the Th2/Th3 cytokines IL-10 and TGF-β (85, 89). A diabetes-retardant hydrolyzed casein diet prevented the expression of biochemical markers of inflammation (62), and upregulated the protective gut mucin content in BB rats (88, 93).

The gut mucosa of patients with T1D is sometimes damaged and can display markers of immune activation such as increased MHC class II expression (83, 90, 94). In the NOD mouse, gut damage was promoted by a wheat-based diet and was characterized by villus atrophy, increased intraepithelial T cells, enhanced epithelial MHC class II levels, and increased IFN-γ, TNF-α, and inducible nitric oxide synthase gene expression (73, 95).

α4β7 is an integrin expressed on lymphocytes which directs their circulation to the gut and to several other tissues. Cells expressing this integrin were among the autoreactive cells infiltrating the pancreatic islets of T1D patients (96) and produced large amounts of IFN-γ and TGF-β (97). α4β7-expressing cells have also been shown to traffic to the pancreas in NOD mice (98), and blocking α4β7 binding prevented T1D (99). Together this suggests that lymphocytes that home to the gut play a role in the pathogenesis of T1D.

The gastrointestinal tract is an important immune tissue (79). It contains the largest collection of immune cells in the body and is required for protection from infection with bacteria, viruses, fungi, and worms encountered in the gut lumen, while maintaining tolerance to self antigens and harmless environmental antigens. The intestinal epithelial cells also secrete cytokines and play a role in immune defence (100). Peyer's patches (PP) are nodules of lymphoid tissue in the gut wall which contain antigen presenting cells (APCs) and

lymphocytes (101). M cells present in these structures sample lumen antigens and present them to lymphocytes. Lymphocytes which encounter their cognate antigen in the PP proliferate, differentiate into fully mature antigen-specific effector cells and migrate through lymphatics to the mesenteric lymph nodes (MLN) where they undergo final maturation. Soluble antigens can also be presented to naïve T cells directly in the MLN either by draining to the MLN with subsequent uptake by resident APCs or via APCs that capture the antigen in the gut and traffic to the MLN (79). MLNs are the major inductive site where dietary antigens are recognized (79). Lymphocytes which mature in the PP and MLN can enter the systemic circulation and migrate to other mucosa-associated lymphoid tissues or can traffic back to the gut.

The gut immune system normally suppresses the response to dietary antigens, resulting in non-responsiveness upon re-exposure to the same antigen. This process is termed oral tolerance (79) and occurs primarily to dietary protein antigens. Establishing oral tolerance to wheat in pre-diabetic BB rats decreased pro-inflammatory IFN-γ production in the gut and reduced diabetes development (62). This finding suggests that dietary wheat protein modification of T1D could involve a breakdown in oral tolerance and an abnormal gut immune response to wheat.

Hypothesis and Objectives

Despite accumulating evidence, little is known about the inflammatory status and immune state of the gut in animals and humans that develop T1D. The central hypothesis that formed the basis of the studies in this thesis was as follows: antigens in wheat protein-based diets could induce inflammatory Th1 cells in genetically susceptible individuals. The main objectives were (i) to develop methods to quantify Th1/Th2 inflammatory cell status in BioBreeding rat tissues, (ii) to use these methods to determine the gut inflammatory status and to identify wheat protein-reactive lymphocytes in diabetes-prone BB rats, (iii) to identify wheat protein-reactive T cells in patients with T1D, (iv) to determine if diabetes incidence is modified by diet in Komeda diabetes-prone (KDP) rats, and (v) to characterize the effect of dietary cereal and of microbes on diabetes development in germ-free BBdp rats.

MATERIALS

Agarose (Sigma-Aldrich Canada, Oakville, ON)

Ammonium chloride (Fisher Scientific, Ottawa, ON)

Bovine serum albumin (Sigma-Aldrich Canada, Oakville, ON)

Casein (Sensient Flavors Canada, Mississauga, ON)

Cell culture Falcon plates (BD Canada, Oakville, ON)

CFSE (Invitrogen Canada, Burlington, ON)

α-chymotrypsin (Sigma-Aldrich Canada, Oakville, ON)

Fetal bovine serum (Sigma-Aldrich Canada, Oakville, ON)

Frosted microslides - 25x75 mm (VWR International, Mississauga, ON)

GeneAmp RNA polymerase chain reaction Kit (Roche Mississauga, ON)

Hepes (Invitrogen Canada, Burlington, ON)

Human serum, pooled (Cedarlane Laboratories, Burlington, ON)

Ionomycin (Sigma-Aldrich Canada, Oakville, ON)

Kodak image station 440CF (Eastman Kodak, Rochester, NY)

L-glutamine (Invitrogen Canada, Burlington, ON)

NTP-2000 diet (Ziegler Bros, Gardners, PA)

One kb plus DNA ladder (Invitrogen Canada, Burlington, ON)

Ovalbumin, albumin protein from chicken egg white (Sigma-Aldrich Canada, Oakville, ON)

PCR tubes (Ultident Scientific, St. Laurent, QC)

Penicillin (Invitrogen Canada, Burlington, ON)

PHA (Sigma-Aldrich Canada, Oakville, ON)

PMA (Sigma-Aldrich Canada, Oakville, ON)

Phosphate buffered saline (Invitrogen Canada, Burlington, ON)

Polypropylene Falcon round bottom 5 mL tubes (BD Canada, Oakville, ON)

Propidium iodide (Sigma-Aldrich Canada, Oakville, ON)

RPMI 1640 medium (Invitrogen Canada, Burlington, ON)

Streptomycin (Invitrogen Canada, Burlington, ON)

Screen cup and 80 μm pore mesh (Sigma-Aldrich Canada, Oakville, ON)

Sodium heparinized glass centrifuge tubes (BD Canada, Oakville, ON)

Tris(hydroxymethyl)aminomethane (Sigma-Aldrich Canada, Oakville, ON)

Trypan blue (Invitrogen Canada, Burlington, ON)

Tween 20 (Sigma-Aldrich Canada, Oakville, ON)

METHODS

Animal Husbandry

Animal studies were performed in accordance with the guidelines of the Canadian Council on Animal Care and the studies were approved by the Health Canada and the University of Ottawa Animal Care Committees. Diabetes-prone BioBreeding (BBdp) rats, a model of spontaneous polygenic T1D, and non-diabetes-prone BB control (BBc) Wistar rats of both sexes were housed at the Animal Resources Division of Health Canada (Ottawa, ON). Animals were maintained in laminar flow-protected cages under specific pathogen-free (SPF) conditions. Tests in sentinel rats indicated the colony was antibody-free with respect to Sendai virus, pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, reovirus type 3, and *Mycoplasma pulmonis*. At 23d, animals were weaned and given free access to their respective diets and water. Diets were either a diabetes-promoting, mainly wheat-based NTP-2000 diet (102), a diabetes-retardant, hydrolyzed casein (HC)-based AIN-93G diet was prepared by enzymatic hydrolysis of casein protein (prepared at Health Canada, Ottawa, ON; 103), or a cereal (CER)-based laboratory Chow 5001 diet (Purina, Mississauga, ON). At the indicated ages, animals were anesthetized with 3 % halothane or isoflurane (Abbott, Montreal, QC) in O_2, weighed, and exsanguinated from the abdominal aorta. Tissues were removed, weighed, and then either analyzed immediately or used to prepare single cell suspensions.

Preparation of Single Cell Suspensions From Rat Tissues

Single cell suspensions were prepared by homogenization of rat spleen tissues between the frosted end of two sterile glass microscope slides in RPMI-1640 and passing the cells through a 70 μm nylon mesh strainer basket (VWR International, Mississauga, ON).

Red blood cells present in spleen preparations were lysed with 5 mL of ice cold erythrocyte lysis buffer (0.144M NH_4Cl, 0.017M Tris-base, pH 7.2), incubated for 10 min at 4 °C, and washed twice with RPMI 1640 medium. Mononuclear cells were then resuspended in the appropriate medium. Cell viability was determined by trypan blue exclusion.

In Vitro Activation of BBc Rat Splenocytes with PMA and Ionomycin

As described in Chakir, et al. (104), non-specific *in vitro* cell stimulation was achieved by culturing BBc rat spleen mononuclear cells (splenocytes) at a density of 1×10^6 cells/mL in RPMI medium in 6-well culture plates with 50 ng/mL PMA and 1 μg/mL ionomycin.

In Vitro Generation of Th1 and Th2 Polarized Cells from BBc Rat Splenocytes

BBc rat splenocytes were seeded in a 24-well plate at 1×10^6 cells/mL in RP-10 medium containing 10 % fetal bovine serum (RPMI 1640 medium supplemented with 25 mM HEPES, 10% (v/v) heat-inactivated FBS (heat-inactivated at 56°C for 1h), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin, pH 7.1) and cultured at 37°C in 5% CO_2. Cell stimulation was carried out as described previously (104, 105). Briefly, to generate Th1 cells, cells were stimulated for 7 days with 5 μg/mL plate-bound anti-rat CD3, 1 μg/mL soluble anti-rat CD28, 10 U/mL human IL-2, 10 ng/mL human IL-12, and 10 μg/mL anti-rat IL-4 mAb. To generate cells with a Th2 phenotype, splenocytes were stimulated with 5 μg/mL plate-bound anti-rat CD3, 1 μg/mL soluble anti-rat CD28, 10 U/mL human IL-2, 100 ng/mL rat IL-4, and 10 μg/mL anti-rat IFN-γ. Cytokine and antibody source is listed in Appendix 1. Unfortunately anti-rat IL-12 antibody, the mouse equivalent of which is used to polarize mouse Th2 cells, was not commercially available and was thus not used. Following 3 days of polarization, cells were

allowed to expand for an additional 4 days by dilution with fresh RP-10 medium and supplementation with 20 U/mL human IL-2. At day 7, primed cells were extensively washed and 1×10^6 cells/mL were re-stimulated with plate-bound anti-rat CD3, soluble anti-rat CD28, and Th1- or Th2-promoting cytokines and antibodies for a second week, following the same procedure as week 1. Following the second week of stimulation, the cells were washed and re-activated with plate-bound anti-rat CD3 and soluble anti-rat CD28 to expand the cell number and to promote gene expression.

Total RNA Isolation

Total RNA was isolated from tissues immediately upon excision, or from single cell suspensions, using TRIzol reagent (Invitrogen Canada, Burlington, ON) according to the manufacturer's protocol. Briefly, following preparation and/or stimulation, cells were centrifuged at 400×*g* for 5 minutes at 4 °C and the pellets were washed twice with PBS. Single cell suspensions were lysed by pipetting $5x10^6$ cells in 1 mL TRIzol reagent. Tissues were homogenized in 2 mL TRIzol reagent using a polytron. Chloroform extraction with centrifugation at 12,000 ×*g* was used to isolate the RNA. The upper aqueous phase, which contained the RNA, was transferred to a fresh tube. Isopropyl alcohol was added to precipitate the RNA. The supernatant was discarded and the pellet was washed with 70% ethanol. The sample was centrifuged at 7500 ×*g* and the pellet was dried. The RNA was resuspended in nuclease-free water and quantified by spectrophotometry. RNA quality was verified by electrophoresis of 2 μg of RNA on agarose gel (1.2 % (w/v) agarose, 20 mM MOPS, 1mM EDTA, 5 mM sodium acetate, 1 % formaldehyde, 0.08 μg/mL ethidium bromide) run in MOPS buffer (20 mM MOPS, 1mM EDTA, 5 mM sodium acetate) and

visualized under UV light. RNA samples were supplemented with 1 % RNase inhibitor and 0.01 M dTT and stored at -80 °C.

Semi-quantitative Reverse Transcription-polymerase Chain Reaction

Reverse transcription (RT) was carried out, as described previously (104), with 2 μg of total RNA, an oligo(deoxythymidine)$_{16}$ primer, and a GeneAmp RNA polymerase chain reaction (PCR) Kit. Briefly, the RNA was denatured for 90 seconds at 60 °C and then cooled to 4 °C. The reverse transcription reaction solution was added (5mM $MgCl_2$, PCR buffer, 2'-deoxynucleoside 5'-triphosphates consisting of dATP, dCTP, dGTP, dTTP each present at 0.5 mM, 40 units RNase inhibitor, 0.1 nmol Oligo-(deoxythymidine)$_{16}$ primer, and 100 units reverse transcriptase). The thermal cycling was performed with an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) as follows: 42 °C for 90 minutes of reverse transcription, followed by 94 °C for 5 minutes to inactivate the reverse transcriptase enzyme.

The PCR reaction with the GeneAmp PCR kit was enhanced with 10 % dimethyl sulfoxide or with the PCRx enhancer system (Invitrogen Canada, Burlington, ON). The PCR reaction solution consisted of 5 μL cDNA, MgCl2, PCR buffer, 3' and 5' primers each present at 0.2 μM, dATP, dCTP, dGTP, and dTTP, each present at 0.125 mM, nuclease-free water and 0.625 units of Taq polymerase. PCR was performed with an Eppendorf MasterCycler. The thermal cycling conditions were as follows: DNA melting at 94 °C for 30 seconds, primer annealing for 45 seconds (temperatures in Appendix 4), DNA synthesis at 72 °C for 60 seconds. These cycles were followed by a 72 °C incubation for 7 min.

PCR primers were designed according to the corresponding structure of the rat, mouse, or human genes. Sense and anti-sense primers were designed to anneal to different exons in order to avoid amplification of contaminating genomic DNA during the reverse transcription (for sequences refer to Appendix 4).

Semi-quantitative RT-PCR was achieved by normalizing gene expression to expression of the β-actin internal control. Annealing temperature and $MgCl_2$ concentration were optimized for each primer set. As described in Chakir, *et al.*,

> "PCR cycle number was optimized for each experimental condition. ... Representative samples were run at different cycle numbers and the optimal cycle number was selected in the region of linearity between cycle number and PCR product intensity. To confirm a linear relationship between template and PCR product intensity at the optimal cycle number, PCR was run at different cDNA concentrations from representative samples. The absence of genomic DNA contamination was confirmed by performing PCR with representative RNA samples without reverse transcription amplification of mRNA" (104).

PCR products were separated by electrophoresis in agarose gel (1.5 % (w/v) agarose, 4.84 g/L Tris-base, 0.11 % (v/v) glacial acetic acid, 2 mM $Na_2EDTA.2H_2O$, 0.1 μg/mL ethidium bromide) run in TAE buffer (4.84 g/L Tris-base, 0.11 % (v/v) glacial acetic acid, 2 mM $Na_2EDTA.2H_2O$, pH 8.8) beside 1 kb plus DNA ladder. The PCR product size (Appendix 4) and the net band intensity were visualized under UV light with a Kodak image station 440CF and analyzed using 1D image analysis software (Appendix Figure 6-2).

Capture ELISA

Cytokine levels in supernatants harvested from *in vitro* cell culture were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (104). Rat cytokine detection was performed using OptEIA rat IFN-γ and IL-4 capture ELISA kits (BD Canada, Oakville, ON). Human cytokine detection was performed using OptEIA capture ELISA kits (BD Canada, Oakville, ON). Recombinant human (rh) IFN-γ, rhIL-4, and rhIL-5

were used as standards. Purified anti-human IFN-γ mAb, purified mouse anti-human IL-4, and purified anti-human IL-5 (TRFK5) were used as capture antibodies. Biotinylated anti-human IFN-γ mAb (4S.B3), biotinylated rat anti-human IL-4, and biotinylated anti-human IL-5 mAb (JES1-5A10) were used at 1μg/ml as detection antibodies.

SDS-PAGE and Western Blot Analysis

Twenty million cells were washed three times in PBS at 4 °C with centrifugation at 800 ×*g* for 5 min. Cell lysates were prepared as previously described (106, 107). Briefly, the pellets were incubated in 250 μL of lysis buffer (10 mM Hepes, 3 mM $MgCl_2$, 10 mM NaCl, 0.1 mM EDTA, 300 mM sucrose, 0.5 mM dithiothreitol and 5 mM phenylmethylsulfonyl fluoride, pH 7.9) for 10 minutes on ice. Following the incubation, 0.1 % (v/v) Nonidet P-40 was added and mixed carefully. The lysate was centrifuged immediately at 800 ×*g* for 1 minute at 4 °C. The supernatant containing the cytosolic protein fraction was removed. The nuclear pellet was lysed by vigorous vortexing in 50 μL of nuclear lysis buffer (20 mM Hepes, 3 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25 % glycerol, 0.5 mM dithiothreitol and 5 mM phenylmethylsulfonyl fluoride, pH 7.9) for 15 minutes on ice. The lysate was centrifuged at 16,000 ×*g* for 10 minutes. The supernatant was removed as the nuclear protein fraction. Protein concentration was determined by Bradford assay with bovine serum albumin (BSA) as the protein standard. BenchMark pre-stained protein ladder (Invitrogen Canada, Burlington, ON) was mixed with an equal volume of sample loading buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer to nitrocellulose membranes. Membranes were incubated with Ponceau S to confirm protein transfer, washed with water, and blocked with 5 % skim milk solution.

For Western blot analysis of rat IFN-γ and IL-4, membranes were probed with monoclonal anti-rat IFN-γ antibody (Invitrogen Canada, Burlington, ON) or with mouse anti-rat IL-4 monoclonal antibody (BD Canada, Oakville, ON) at a concentration of 1:1000. For Western blot analysis of rat T-bet and GATA-3, membranes were probed with monoclonal mouse IgG_1 anti-T-bet antibody (4B-10) at a concentration of 1:200 for 1.5 h, or with mouse IgG_1 anti-GATA-3 antibody (HG3-31) at a concentration of 1:125 for 1.5 h. The anti-T-bet antibody was kindly provided by Dr. Susanne Szabo (Harvard Medical School, Boston, MA). Secondary antibody was HRP-conjugated rabbit anti-mouse IgG antibody, applied at a concentration of 1:1000 for 1 h. Western blots were developed with the ECL system. Mean band intensity was quantified using a Kodak image station 440CF and analyzed using 1D image analysis software.

Flow Cytometric Phenotyping

Flow cytometry was used to determine cell surface marker phenotype. $1x10^6$ cells/measurement were resuspended in 100 μL FACS buffer (1 % (w/v) BSA, 0.1 % (w/v) NaN_3, in PBS, pH 7.4) and stained with fluorochrome-conjugated antibodies (Appendices 2 and 3) for 30 min. Cells were washed twice with FACS buffer followed each time by centrifugation at 350 ×*g* for 5 minutes at 4°C. The percentage of cells expressing the markers was analyzed with a FC500 flow cytometer (Beckman-Coulter, Mississauga, ON). Isotype controls antibodies were used to control for non-specific staining. A minimum of $2x10^4$ events was analyzed. Following analysis by flow cytometry, cells were re-stained with 1 μg of propidium iodide. Dead cells were excluded from the analysis by gating on propidium iodide-negative cells.

Preparation of Wheat Protein Antigens

Wheat proteins (WP) were extracted from wheat gluten powder (ICN Biochemicals, Cleveland, OH) using partial hydrolysis with chymotrypsin as described previously (108). Briefly, as described in a paper on which I share first authorship (109), wheat gluten was partially digested for solubilization with 100:1 (w/w) α-chymotrypsin (Sigma-Aldrich Canada, Oakville, ON) in 174 mM Tris base, pH 7.8, for 24 h at 37°C. The digested wheat protein was centrifuged at 1,500 ×*g* for 15 min at 22°C and then filtered through a 0.2 μm Acrodisc filter (Millipore, Billerica, MA) to remove large particles. Protein concentration was determined using the Bradford assay with BSA as the protein standard.

CFSE Labeling

Single cell suspensions from rat tissues were resuspended in sterile PBS at $20x10^6$ cells/mL. Human peripheral blood mononuclear cells (PBMC) were resuspended at a concentration of $20x10^6$ cells/ml in HBSS medium containing 20 mM Hepes. Rat cells were stained with 5 μM CFSE. Human cells were stained with 2 μM CFSE. Cells were incubated with CFSE for 20 minutes at 37°C with gentle shaking every 5 min. Rat cells were then washed twice with RP-10 medium. Human cells were washed twice with ice-cold HBSS medium containing 20 mM Hepes and 4% (v/v) heat-inactivated pooled human serum (Biological Specialty Corporation, Colmar, PA).

CFSE-based Flow Cytometric Proliferation Assay with Rat Cells

MLN and spleen cells were seeded in a 24-well plate at 2×10^6 cells/mL in RP-10, in the presence of α-chymotrypsin-treated wheat protein (12.5-50 μg/mL) or control treatments, and cultured at 37°C in 5% CO_2. As described by Turcanu, *et al.* (110), cells cultured with medium alone or treated with chicken egg white ovalbumin (OVA) protein served as

negative controls. As a positive control for T cell proliferation, cells were cultured with 5 μg/ml plate-bound anti-CD3 antibody and 1 μg/ml soluble anti-CD28 antibody (reagent source in Appendix 1). Anti-CD3 was bound to the plate by 90 minute incubation at 37°C in PBS without Ca^{2+} or Mg^{2+}. Non-CFSE-stained cells were cultured with the same conditions, to be used for flow cytometry autofluorescence and compensation. Beginning on day 3-5, cells were supplemented with 10 U/mL human IL-2, to promote the expansion of proliferating cells, and split to maintain a density of 2×10^6 cells/mL. At 3 and 7 days after stimulation, cells were harvested and stained with cy-chrome-conjugated anti-rat CD4 and PE-conjugated anti-rat αβ-TCR antibodies (Appendix 3) for analysis by flow cytometry. The response to antigen was reported as the ratio of $CD4^+CFSE^{low}$ cells to total $CD4^+$ cells analyzed. $CD4^+$ MLN cells which proliferated in response to wheat protein ($CD4^+CFSE^{low}$) were analyzed for αβ-TCR expression, a specific marker of a major subset of CD3 T cells (109).

Preparation and Storage of Human PBMCs

Studies were approved by the Ottawa Hospital Research Ethics Board. PBMCs were obtained with informed consent from patients and controls. Blood was collected in heparin-treated vacutainer tubes and diluted 1:1 in sterile PBS. PBMC were isolated by centrifugation for 30 minutes at 400 ×*g* at 22°C on a Histopaque-1077 density gradient (Sigma-Aldrich Canada, Oakville, ON). Cells were quantified by trypan blue exclusion assay. To freeze and store freshly isolated PBMCs and purified lymphocytes, cells were resuspended at $5x10^6$ cells/mL of freezing medium (90 % (v/v) heat-inactivated FBS, 10 % (v/v) DMSO), and stored in liquid nitrogen. Frozen cells were reconstituted by thawing the vial in a 30 °C water bath and washing twice with 15 mL RPMI.

Generation of Antigen-specific T cell Lines with PBMCs from a Patient with T1D and CD

For primary stimulation, PBMCs from the patient with T1D and CD and from healthy control subjects (n=3) were plated in 12-well plates at 3.5×10^6 PBMCs/mL and stimulated in Xvivo medium with α-chymotrypsin-treated, heat-inactivated wheat gluten protein (WP; 0–25 μg/mL), α-gliadin 33-mer wheat peptide (0-10 μM), the diabetes-related autoantigen human insulin protein (0-100 μg/mL; Sigma-Aldrich Canada, Oakville, ON), or recombinant diabetes-related autoantigen GAD protein (0-50 μg/mL; Sigma-Aldrich Canada, Oakville, ON). The α-gliadin 33-mer peptide (residues 57 to 89; Ref. 111) was provided by Dr. Hubert Kolb (German Diabetes Research Institute, Dusseldorf, Germany). As negative controls, cells were incubated with medium alone or in the presence of chicken egg OVA protein (OVA, 44 μg/mL). As a positive control, cells were incubated with the mitogen phytohemaglutinin (PHA; 2.5-5 μg/mL). Cells were incubated at 37°C in 5% CO_2. After 2-5 days, 10 U/mL of human IL-2 was added. The cells were allowed to divide for 14 days. To restimulate activated T cells without inducing apoptosis, they must first be treated to return them to a resting state characterized by low level cytokine secretion and a round shape. The proliferating T cells from the patient with T1D and CD were rested by culturing for 6 d in fresh medium containing 1 U/mL human IL-2 and 10 ng/mL IL-7 (reagent source in Appendix 1). Cells were then resuspended in freezing medium, and frozen in liquid nitrogen. Freezing also rests T cell lines and ensures that T cells which don't express IL-7 receptor are also rested (112). Cells were thawed, plated *in vitro* at 2×10^6 cells/mL, and subjected to re-stimulation for 1 day with 3.1 μg/mL WP in the presence of 2×10^6 cells/mL irradiated (30 Gray) autologous PBMC, which act as functional, but non-dividing antigen presenting cells (APC).

CFSE-based Cytometric Proliferation Assay with Human PBMCs

A CFSE-based proliferation assay was performed as described previously (110) to determine immune cell reactivity. Briefly, CFSE-labeled human PBMCs were resuspended in RP-5H culture medium containing 5% human serum (RPMI-1640 medium containing 25 mM HEPES, 5 % (v/v) heat-inactivated pooled human serum, 50 μM 2β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, pH 7.1) at 1.5×10^6 cells/mL in 24 well plates (2 mL per well). Cells were cultured with or without α-chymotrypsin-treated wheat gluten protein (WP) at varying concentrations (3.1, 6.25, 12.5 μg/mL) for 10 days. As a negative control, cells were cultured with 44 μg/mL OVA protein. As positive control for proliferation, cells were incubated in the presence of 2.5 μg/ml PHA. Non-CFSE-stained cells were cultured with 6.25 μg/mL WP or 2.5 μg/mL PHA to be used for flow cytometry autofluorescence and compensation. Beginning at day 3-5 post-stimulation, cells were supplemented with 10 U/ml human IL-2 and split to maintain a cell density of 1.5×10^6 cells/mL. At d3 and d7, cells were harvested and stained with SPRD-conjugated anti-CD3 mAb (source in Appendix 2) for flow cytometry analysis. Data were reported as the percentage of proliferating $CD3^+$ T cells, exhibiting low CFSE staining ($CD3^+CFSE^{low}$), in the total population of lymphocytes.

MoFlo Sorting of Human Cells

CFSE-stained PBMCs cultured with WP for 11-12 days were stained with SPRD-conjugated anti-human CD3, and WP-reactive cells ($CD3^+CFSE^{low}$) were sorted at 90-99% purity using Cytomation Mo-Flo Cell Sorter. Cells were then resuspended in freezing medium and frozen in liquid nitrogen to rest them.

Re-stimulation of Sorted Wheat Protein-reactive Human T Cell Lines

WP-specific T cell lines were generated by plating 5 x 10^4 cells/well of sorted, WP-reactive T cells in 96-well U-bottom plates with 2 x 10^5 irradiated (30 Gray) autologous PBMC, to act as APC, in a total volume of 200 µL RP-15H medium containing 15% human serum (RPMI-1640 medium containing 25 mM HEPES, 15 % (v/v) heat-inactivated pooled human serum, 50 µM 2β-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine, pH 7.1). Non-sorted WP-specific T cell lines were also generated by plating 1x10^6 cells/mL non-sorted, WP-stimulated T cells in RP-15H medium in 24-well plates, with irradiated autologous PBMC as APCs. Cells were stimulated in the absence or presence of varying concentrations of chymotrypsin-treated WP (1.5-6.25 µg/mL). Cells were also incubated in the presence of β-lactoglobulin as a negative control or were stimulated with 2.5 µg/ml PHA to test proliferative capacity. As a control for background, irradiated APCs or T cells were cultured alone or in the presence of WP antigens. Cells plated in 24-well plates were supplemented with 10 U/mL human IL-2 and split to maintain a cell density of 1×10^6 cells/mL. Cell health and activation was observed by light microscopy.

Thymidine Incorporation Proliferation Assay

Antigen-stimulated wheat protein-reactive T cell lines plated in 96-well plates (described above) were pulsed for 24 h with 1 µCi per well of [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ). Cells were harvested onto glass fiber filters and incorporated thymidine radioactivity was measured as counts per minute (cpm) on a Matrix β-particle counter (Hewlett-Packard Company, Palo Alto, CA).

Genotyping of KDP Rats

The KDP rat genotype was determined by PCR-restriction fragment length polymorphism analysis of the Cbl-b gene as described previously (113, 114). Briefly, rat tails were cut at 0.5 cm and genomic DNA was prepared using the DNeasy Tissue Kit (Qiagen, Mississauga, ON) and was quantified by spectrophotometry. The Cbl-b gene was amplified from 1 μg of genomic DNA by PCR using the GeneAmp PCR kit. Cbl-b primers were as follows: TGCCCCTTCTGTCGCTGTGA and CCTCGGTTTTGAATCAACAG and the annealing temperature was 55°C. PCR products were digested with 2 units of TaqI restriction enzyme (Invitrogen Canada, Burlington, ON) at 65°C for 1 h. Digests were separated by electrophoresis in 4 % agarose gel beside 1 kb plus DNA ladder and the PCR product size was analyzed using a Kodak image station 440CF. The wild-type Cbl-b gene product is cleaved by TaqI restriction digest, resulting in a 160 bp band, while the polymorphism in the mutant Cbl-b gene in KDP rats disrupts the TaqI enzyme site, resulting in a 182 bp band.

Diabetes Monitoring

Beginning at 55 days of age, animals were tested twice weekly for glucosuria. Those with a value greater than 2+ were fasted overnight, and glucose in tail blood was measured the next morning using a glucometer. Diabetes was diagnosed when fasting blood glucose was >11.1 mmol/liter.

Derivation and Maintenance of Germ-free BBdp Rats

Germ-free (GF) BBdp rats were derived from SPF rats by caesarean section at Health Canada. Briefly, term SPF BBdp mothers were anesthetized, and a hysterectomy was performed. The uterus was clamped, dipped in Clidox solution (Fisher Scientific, Ottawa,

ON) for sterilization and placed in sterile isolators. GF pups were nursed by GF Sprague Dawley foster mothers from (IFFA-CREDO, Lyon, France); distributed by Charles River (Montreal, QC).

A GF environment was maintained in plastic shoebox cages with isolator caps inside sterile closed isolator hoods maintained under positive pressure and equipped with fans to circulate HEPA-filtered air (Charles River, Wilmington, MA). Sterile wood bedding and sterile irradiated water were used (Charles River, Wilmington, MA). GF rats were weaned at 23 d onto either an irradiated AIN93G hydrolyzed casein (HC) diet or an irradiated cereal (CER)-based C.R. Rodent 18% Vac-Pac diet (PMI Nutrition International, Brentwood, MO). These diets had been vacuum packed in 5 pound heat-sealed barrier bags (Zeigler Bros, Gardners, PA), sterilized by gamma irradiation with a minimum dose of 34.5 kGy and a maximum dose of 46.6 kGy at (MDS Nordion, Montreal, QC) and stored at 4°C. Samples of untreated and irradiated diets were evaluated by Health Canada for bacterial contamination by enrichment culturing, dilution plating and incubation of the plates under aerobic and anaerobic conditions. Following the gamma irradiation, no culturable aerobic or anaerobic bacteria were detected. Diets were analyzed for thiamine-HCl by Silliker Canada (Markham, ON). Dietary thiamine, the vitamin which is most susceptible to inactivation by radiation damage, was partially degraded by the irradiation process (115). Thus irradiated diets were supplemented with 30 % more vitamin mix prior to irradiation. Before use, isolators and cages were sterilized by gas fumigation with a solution of 5.7 % peracetic acid and 1.15 μg/mL nacconal. Supplies were soaked in bactericidal, virucidal, fungicidal Clidox solution (Pharmacal Research Labs, Naugatuck, CT) for sterilization before placing in the port of the isolator. Sterile items and animals were asceptically transferred to the GF isolators via a

transfer sleeve and port under positive pressure. For the course of the experiment, animals were handled through arm ports with gloves secured by a sleeve clamp. The virus-free status of GF rats was confirmed by ELISA for viral antibodies against Sendai virus, pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, Theiler's virus GDV-2, reovirus type 3, Mycoplasma pulmonis, lymphocytic choriomeningitis virus, mouse adenovirus 1 & 2, hantavirus hantaan, Encephalitozoon cuniculi, cilia-associated respiratory bacillus, mouse parvovirus NS1, rat parvoviruses, rat miute virus, and by indirect immunofluorescent assay and hemagglutination inhibition titers for Kilham rat virus performed at Charles River Diagnostics (Wilmington, MA).

Microbiological Monitoring of Germ-free Rats

Routine testing by the Animal Resources Division at Health Canada confirmed that bacteria were absent in GF rats. Briefly, fresh fecal pellets and environmental samples from the isolation chambers were routinely monitored for the presence of viable bacteria. Samples were incubated in peptone water at 37°C for 24 h and aliquots were distributed and cultured on Plate Count agar, Tryptone Bile agar, Baird Parker agar, Hektoen agar, Sabouraud-Dextrose agar, or McConkey agar (116). These detect the growth of gram negative and gram positive aerobic and anaerobic bacteria.

Many commensal bacteria species cannot be cultured by conventional techniques, thus DGGE was performed at Health Canada to confirm the GF status (117, 118). Bacterial genomic DNA was isolated from fecal samples using the QIAmp DNA Stool Mini kit (Qiagen, Mississauga, ON). Genes encoding bacterial 16S ribosomal RNA were amplified using the universal primers F27 and R1492, as previously described (119). Isolates were compared by electrophoresis of the PCR amplicons on 40-60% denaturing gradient

electrophoresis gels (120). Samples from GF rats yielded no PCR products, confirming the absence of bacteria.

Preparation of Fixed Tissue Sections

Isolated tissues were immersed in Bouins fixative solution. Gut jejunum tissues were cut open longitudinally, rinsed with PBS, and rolled up prior to fixation. Following 24 h, all tissues were transferred to 70 % ethanol. Tissues were dehydrated and embedded in paraffin. Sections 5 μm thick were cut, placed on silanized slides, and deparaffinized in xylene or citrosolv solutions.

Hematoxylin and Eosin Staining

Fixed tissue sections were rehydrated and stained with hematoxylin for 2 min then rinsed with water. Hematoxylin staining was developed with saturated lithium chloride then rinsed with water. Sections were then stained with eosin for 2 min and rinsed with water.

Islet Number Evaluation

All histological analyses were performed on coded samples. Total islet number was evaluated on one hematoxylin- and eosin-stained section per animal using a Zeiss Axiolab light microscope. Section area was scanned using an Axioplan 2 light microscope (Zeiss Canada, Mississauga, ON) at 100× and a QImaging color camera. A "SuperImage" of the entire slide was compiled and total section area was measured using Northern Eclipse morphometry software (Empix Imaging, Mississauga, ON). Fat, mesentery, and pancreatic lymph node area was excluded from the analysis. Total islet number was normalized by expression as the number per cm^2 section area.

Insulitis Evaluation

Insulitis was evaluated on hematoxylin and eosin-stained sections of pancreas at a magnification of 100× and was confirmed at 200× magnification using an Axiolab light microscope (Zeiss Canada, Mississauga, ON). A subjective overall rating of islet inflammation/damage (containing infiltration of 5 or more mononuclear cells) was performed as previously described to determine the extent of insulitis (121). The percentage of islets with mononuclear cell peri-insulitis, mononuclear cell infiltration and/or end-stage atrophic damage, was scored on one section per animal.

Tubular Complex Evaluation

Tubular complexes were identified as distinct structures composed of acinar and/or islet tissue containing clusters of more than four ducts. The percentage of rats with one or more tubular complexes was determined on one section per rat as described by Wang, *et al* (121).

Immunohistochemical Detection of Insulin with Fixed Tissue Sections

For insulin analysis, 5 μm paraffin sections were treated with CitriSolv, re-hydrated, and stained for insulin as described previously (121). Briefly, sections were incubated with 3% H_2O_2 in methanol to quench endogenous peroxidases and were blocked with Universal Blocking Solution (DAKO Canada, Mississauga, ON). Guinea pig anti-porcine insulin (DAKO Canada, Mississauga, ON) was applied at 1:150 and incubated for 2 hours at 22 °C as the primary antibody followed by incubation with biotinylated rabbit anti-mouse IgG (1:300) for 30 min (DAKO Canada, Mississauga, ON) as the secondary antibody. Pre-formed streptavidin-biotin complex conjugated with horseradish peroxidase was incubated with streptavidin-horseradish peroxidase to increase the detection sensitivity (DAKO

Canada, Mississauga, ON). This was then applied to the slides and incubated at 22°C for 30 min. A brown color was developed in 5 min with 0.06 % diaminobenzidine (DAB; Sigma-Aldrich Canada, Oakville, ON) and 0.03 % H_2O_2 as substrates. For all assays, omitting the primary or secondary antibody resulted in no staining.

Statistical Analyses

Statistical analyses were performed with Statistica, version 6.0 (StatSoft, Tulsa, OK). Comparisons between two sample populations were made using the *t* test for independent variables. Comparisons between multiple sample populations were made using one-way analysis of variance (ANOVA) along with the least significant difference (LSD) post-hoc test to confirm the significance of differences between multiple means. Survival analysis using the log Rank test was used to compare the effect of different treatments on diabetes incidence. A *p* value less than 0.05 was considered significant.

CHAPTER 1 – Ratio of the T cell-specific Transcription Factors T-bet/GATA-3 as a Measure of T helper 1 / T helper 2 Cytokine Status

CHAPTER 1 – INTRODUCTION

When the T cell receptor (TCR) on a naïve CD4 T cell recognizes cognate antigen in the presence of polarizing cytokines, the T cell differentiates into a Th1 or a Th2 effector cell. These cells are identified not by surface markers, but rather by the cytokines they secrete (5, 18). Th1 cells produce a large amount of IFN-γ (6) and promote cell-mediated immune defense against intracellular pathogens. They also play a significant role in autoimmune diseases such as type 1 diabetes (T1D; 122, 123). In addition to playing a role in atopic as well as allergic reactions, Th2 cells defend the host against extracellular parasites. IL-4, which is a major Th2 cytokine, can be measured in early Th2 development. Other Th2 cytokines include IL-5, IL-10 and IL-13 (6, 17; Table 1-1).

Upon T cell stimulation, the cytokines present in close proximity to the cell are together the most important factor determining its differentiation. The differentiation of naïve CD4 T cells towards Th1 or Th2 cells is regulated by the transcription factors T-box expressed in T cells (T-bet; Ref. 7) and GATA-binding protein-3 (GATA-3; Ref. 124), respectively. These transcription factors promote the expression of Th1 or Th2 cytokine genes and play a crucial role in CD4 T cell differentiation. T-bet inhibits Th2 cell differentiation and most importantly initiates Th1 development (7). GATA-3 inhibits Th1 cell differentiation and is a crucial component for the development of the Th2 phenotype (124, 125).

It was hypothesized that the balance between T-bet and GATA-3 expression is representative of the balance between Th1 and Th2 cytokines, and may provide a useful tool to define the phenotype of mixed populations of immune cells.

CHAPTER 1 – EXPERIMENTAL DESIGN

To generate Th1 and Th2 cells, naïve BioBreeding control (BBc) Wistar rat splenic mononuclear cells (splenocytes) were stimulated under Th1 or Th2 conditions. These cells were used to determine if the T-bet/GATA-3 gene and protein expression ratio reflects Th1/Th2 cytokine status. The surface marker phenotype of lymphocytes stimulated under Th1 or Th2 conditions was established by flow cytometry.[a]

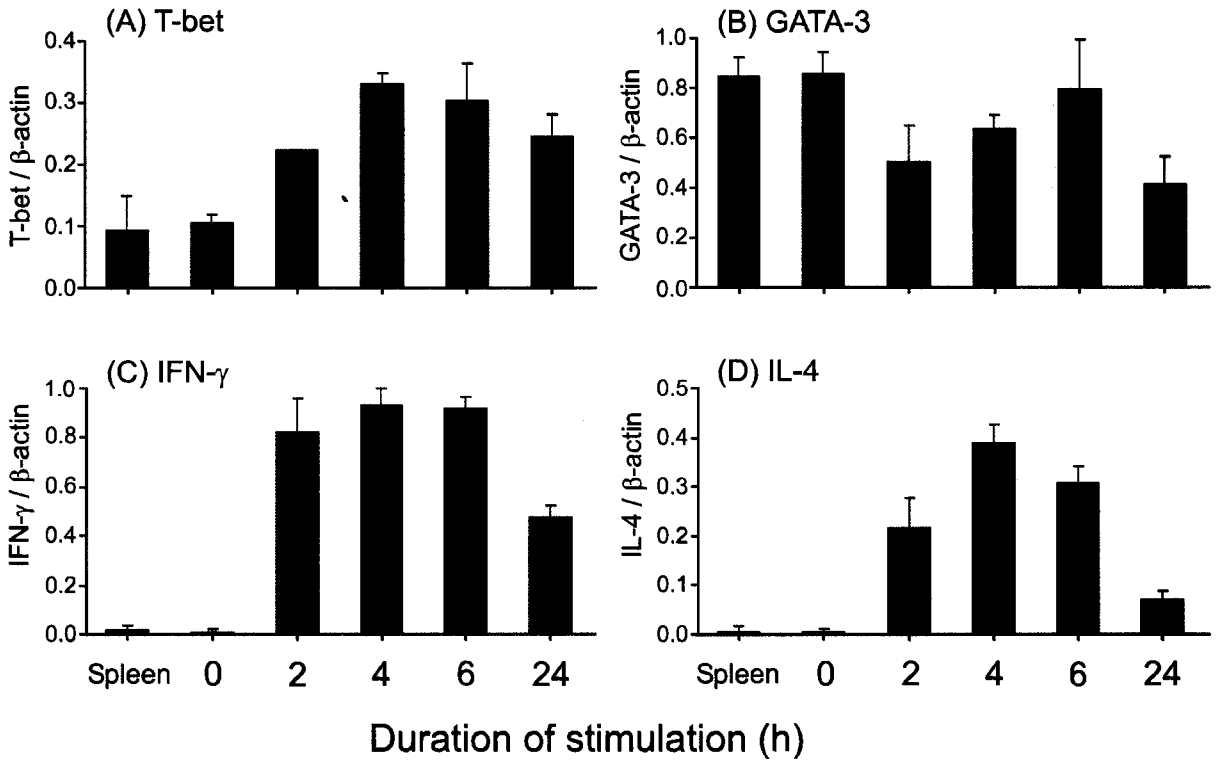
[a] The following sections contain some information which is included in the following publications on which I am a co-author: (104) Chakir, H., H. Wang, D.E. Lefebvre, J. Webb, and F.W. Scott. 2003. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. *J Immunol Methods* 278:157-169. (126) Wang, H., D.E. Lefebvre, and F.W. Scott. 2002. T-bet/GATA-3 gene expression as a measure of Th1/Th2 status in mesenteric lymph nodes and Peyer's patches of BB rats (Abstract). *Society for Mucosal Immunology - Mucosal Immunology Update* 10:6021.

CHAPTER 1 – RESULTS

T-bet and GATA-3 mRNA expression was measured in freshly isolated spleen tissue, in freshly prepared rat splenocyte single cell suspensions, and in PMA+ionomycin stimulated spleen cells to compare gene expression levels by semi-quantitative RT-PCR. The levels of mRNA, normalized to β-actin, are expressed as arbitrary units. As previously demonstrated (14), T-bet was expressed at a low level in spleen tissue as well as in resting splenocytes (Fig. 1-1A). PMA+ionomycin stimulation upregulated the expression of T-bet following 2h, and expression peaked between 4–6 h. In agreement with previous reports (8, 127), high GATA-3 expression was observed in whole spleen as well as in resting splenocytes (Fig. 1-1B). GATA-3 expression was maintained during the PMA+ionomycin stimulation. IFN-γ expression correlated with T-bet expression (Fig. 1-1C). Peak IFN-γ expression was observed at 4-6 h. PMA+ionomycin stimulation also induced IL-4 gene expression (Fig. 1-1D). Comparison of whole spleen tissue with isolated splenocytes indicates that the isolation procedure did not change gene expression levels.

Figure 1-1. T-bet, GATA-3 and cytokine gene expression before and after *in vitro* PMA+ionomycin stimulation

Spleen tissue (Spleen) or splenocytes (0 h) from 60 day, cereal-fed, male control BB (BBc) rats (n=2) were stimulated *in vitro* with PMA and ionomycin for 0, 2, 4, 6 and 24 h. Gene expression was measured by RT-PCR for T-bet (A), GATA-3 (B), IFN-γ (C) and IL-4 (D) in duplicate samples from each rat. Data were normalized to β-actin expression and represent the mean±SD for two rats. Modified and reprinted from *Journal of Immunological Methods* vol. 278, pp. 157-169, 2003, with permission from Elsevier.


(A) T-bet
T-bet / β-actin
0.0
0.1
0.2
0.3
0.4
(B) GATA-3
GATA-3 / β-actin
0.0
0.2
0.4
0.6
0.8
1.0
(C) IFN-γ
IFN-γ / β-actin
0.0
0.2
0.4
0.6
0.8
1.0
(D) IL-4
IL-4 / β-actin
0.0
0.1
0.2
0.3
0.4
0.5
Spleen
0
2
4
6
24
Duration of stimulation (h)

Expression of T-bet has been shown to be induced specifically in purified developing Th1 clones (7), whereas GATA-3 expression is selectively induced in Th2 clones (8, 125). To test whether T-bet/GATA-3 ratio reflects Th1 and Th2 cytokine levels in mixed populations, T-bet and GATA-3 gene and protein expression was measured in BBc splenic mononuclear cells stimulated *in vitro* under conditions that promote Th1 or Th2 cell differentiation. T-bet was up-regulated after stimulation under Th1 conditions during day 1–3 (Fig. 1-2A). Following that, T-bet expression was maintained constant in Th1 cells. In contrast, T-bet was downregulated in splenocytes stimulated under conditions which favour Th2 differentiation. GATA-3 expression was maintained during the first week of Th1 and Th2 development with higher expression in Th2 cells (Fig. 1-2B). The Th1/Th2 polarizing treatment correlated more closely with the T-bet/GATA-3 ratio than with T-bet or GATA-3 expression alone (Fig. 1-2C). This ratio was consistently high under Th1 conditions and low under Th2 conditions. At day 7, the increased T-bet/GATA-3 ratio in Th1 cells correlated with high expression of the Th1-specific cytokine IFN-γ (Fig. 1-2D). IFN-γ protein expression, analyzed by Western blot, was upregulated during the first two days of the Th1 stimulation procedure (104). The decreased T-bet/GATA-3 ratio in Th2 cells correlated with gene and protein expression of the Th2-associated cytokine IL-4 (Fig. 1-2E; Ref. 104).

The determination of gene and protein expression was repeated at day 7 of the Th1 and Th2 stimulation protocols (Figures 1-3 and 1-4). As in Figure 1-2A, T-bet gene expression was increased in Th1 and reduced in Th2 cells at day 7 (Fig. 1-3A). T-bet mRNA was not translated to protein on day 0, but was upregulated in Th1 compared with Th2 cells following the first week of stimulation (Fig. 1-4A). Although GATA-3 mRNA expression was maintained slightly higher than baseline following the first week of stimulation (Figures 1-3B, 1-2B), GATA-3 protein was higher in Th2 than in Th1 cells (Figure 1-4B). T-bet and GATA-3 protein expression (Fig. 1-4) reflected gene expression of the appropriate cytokine (Fig. 1-3D,E).

Figure 1-2. T-bet, GATA-3 and cytokine gene expression during the early stages of *in vitro* Th1 or Th2 differentiation

Splenocytes were purified from 60 day, cereal-fed, male control BBc rats (n=2). Cells were cultured *in vitro* with antibodies and cytokines which polarize cells towards a Th1 or a Th2 phenotype for 0, 1, 3, 5, and 7 d, and were compared with non-stimulated splenocytes (day 0). Gene expression was measured by RT-PCR for T-bet (A), GATA-3 (B), IFN-γ (D) and IL-4 (E) in duplicate samples from each rat. Data were normalized to β-actin expression. (C) T-bet/GATA-3 ratio for figures A and B. Data are expressed as the mean±SD for two rats. * indicates a statistically significant difference (p<0.05) between Th1 and Th2 differentiation as determined by ANOVA followed by post-hoc analysis. Modified and reprinted from *Journal of Immunological Methods* vol. 278, pp. 157-169, 2003, with permission from Elsevier.

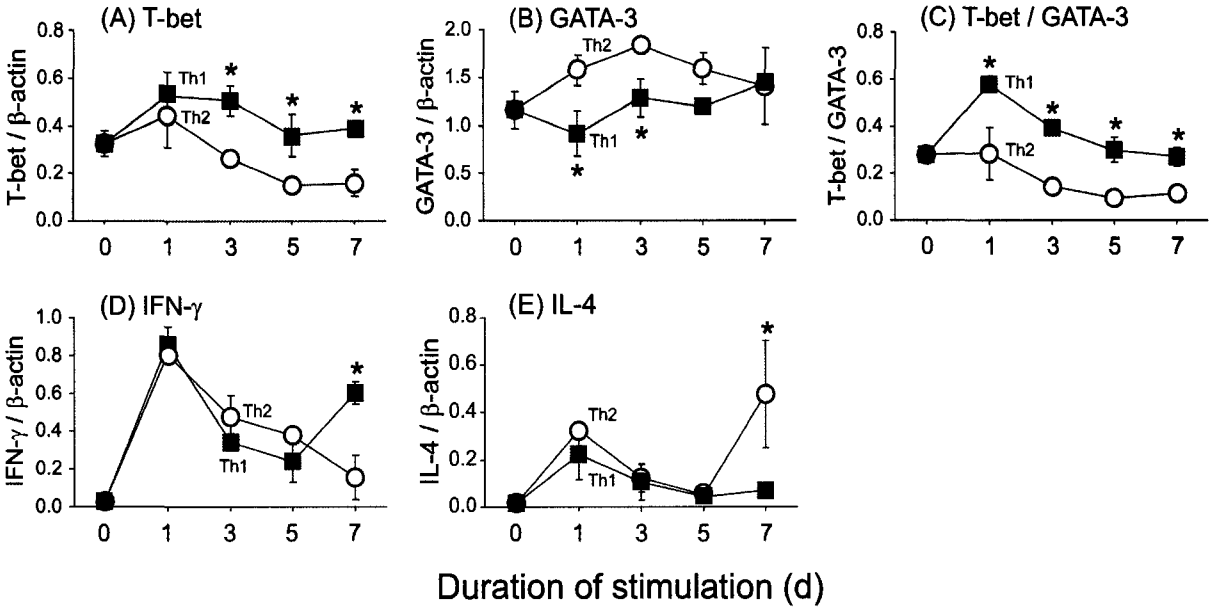

(A) T-bet
T-bet / β-actin
Th1
Th2
(B) GATA-3
GATA-3 / β-actin
(C) T-bet / GATA-3
T-bet / GATA-3
(D) IFN-γ
IFN-γ / β-actin
(E) IL-4
IL-4 / β-actin
Duration of stimulation (d)

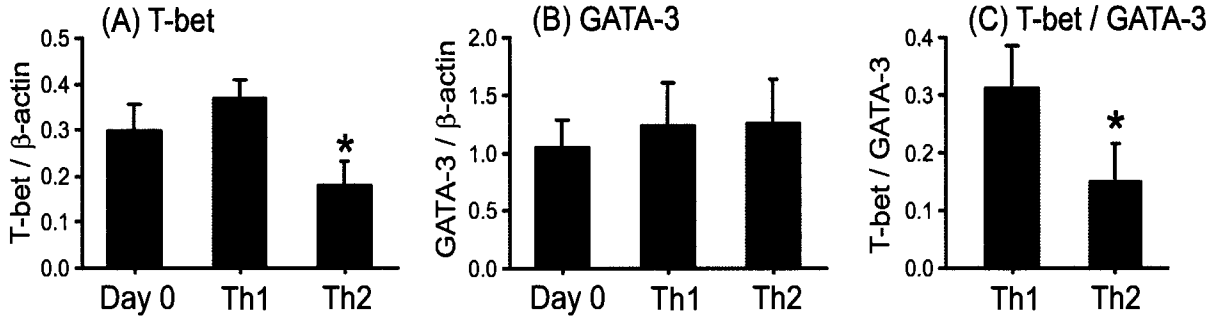
Figure 1-3. T-bet, GATA-3, and cytokine gene expression following one week of *in vitro* Th1 or Th2 differentiation

Splenocytes were purified from 60 day, cereal-fed, male control BBc rats (n=3). Cells were cultured *in vitro* with antibodies and cytokines which polarize cells towards a Th1 or a Th2 phenotype for 7d, and were compared with non-stimulated splenocytes (Day 0). Gene expression was measured by RT-PCR for T-bet (A), GATA-3 (B), IFN-γ (D) and IL-4 (E) in duplicate samples from each rat. Data were normalized to β-actin expression. (C) T-bet/GATA-3 ratio for figures A and B. (F) IFN-γ/IL-4 ratio for figures D and E. Data are expressed as mean±SD for three rats. * indicates a statistically significant difference (p<0.05) between Th1 and Th2 differentiation as determined by ANOVA followed by post-hoc analysis.

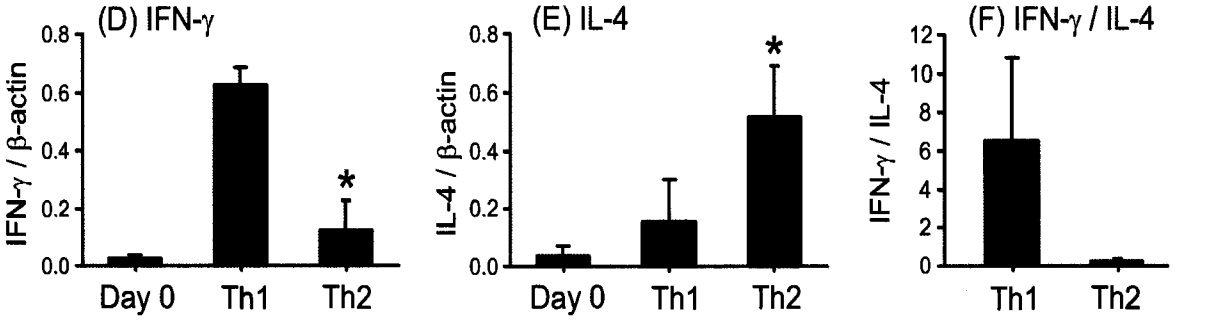
Figure 1-4. T-bet and GATA-3 protein expression following one week of *in vitro* Th1 or Th2 differentiation

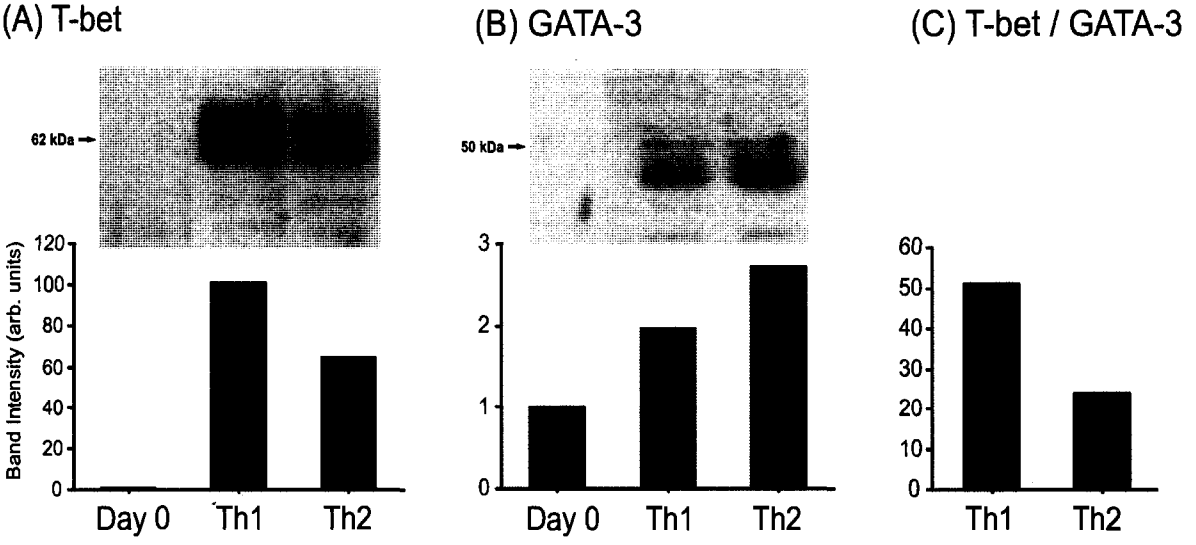
Splenocytes were purified from 60 day cereal-fed, male control BBc rats (n=2). Cells were cultured *in vitro* with antibodies and cytokines which polarize cells towards a Th1 or a Th2 phenotype for 7 d, and were compared with non-stimulated splenocytes (day 0). Five μg of nuclear protein was separated on a 10% polyacrylamide gel with a 6 % stacking gel, and protein expression was measured by immunoblot for T-bet (A) and GATA-3 (B). (C) T-bet/GATA-3 ratio for figures A and B. Data are expressed as mean intensity corrected for background, and are representative of two experiments.

Transcription Factor Gene Expression



Cytokine Gene Expression





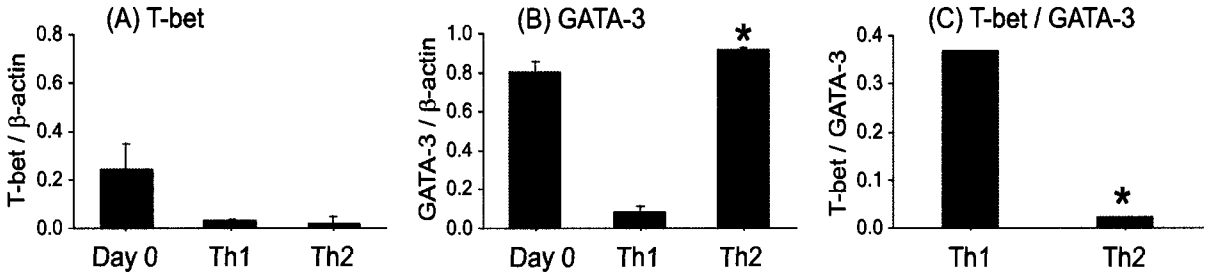
The T-bet/GATA-3 ratio and cytokine profile was determined in long-term fully polarized Th1 and Th2 rat splenocytes (Figure 1-5). These cells were obtained by stimulating the T cells under Th1 or Th2 conditions for one week, repeating the same stimulation condition for a second week and then reactivating the T cells for one additional day. Contrary to the status during early polarization, after long-term *in vitro* stimulation, both Th1 cells (which secreted IFN-γ [Fig. 1-5G]) as well as Th2 cells (which secreted IL-4 [Fig. 1-5H]) expressed little T-bet (Fig. 1-5A). GATA-3 expression was maintained in Th2 cells and was down-regulated in Th1 cells (Fig. 1-5B), which correlated with IL-4 protein in Th2 cells (Fig. 1-5H). The Th1 and Th2 cytokine status in fully polarized cells was better reflected by the T-bet/GATA-3 ratio, which was increased in Th1 and decreased in Th2 cells (Fig. 1-5C). This ratio also correlated with the IFN-γ/IL-4 ratio at the mRNA and protein level (Fig. 1-5F,I).

The phenotype of the T cell populations present during the course of the Th1 and Th2 stimulation of control rat splenocytes was determined by flow cytometry (data not shown). Following the initial stimulations, CD4 and CD8 T cells represented 80-90 % of the cells present. CD8 T cells were less frequent following Th2 compared with Th1 stimulation and they expanded slower than CD4 T cells under both conditions.

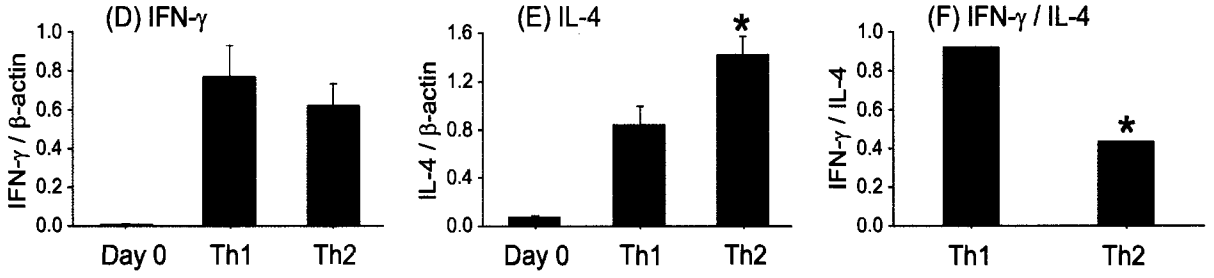
Figure 1-5. T-bet, GATA-3 and cytokine gene expression in long-term polarized Th1 and Th2 cells

Splenocytes were isolated from 60 day, cereal-fed, male control BBc rats (n=2). Cells were cultured *in vitro* with antibodies and cytokines which polarize cells towards a Th1 or a Th2 phenotype. Cells were expanded for one week and were re-stimulated under Th1 or Th2 conditions for a second week. The polarized Th1/Th2 cells were then stimulated for one additional day with plate-bound anti-CD3 and soluble anti-CD28. Gene expression was measured by RT-PCR for T-bet (A), GATA-3 (B), IFN-γ (D), IL-4 (E). Data were normalized to β-actin expression and represent mean±SD in duplicate samples. (C) T-bet/GATA-3 ratio for figures A and B. (F) IFN-γ/IL-4 ratio for figures D and E. Cell-free supernatants were recovered following two days of the third stimulation for IFN-γ (G) and IL-4 (H) cytokine protein secretion analysis by capture ELISA. Data represent the mean±SD for two rats. (I) IFN-γ/IL-4 ratio for figures G and H. * indicates a statistically significant difference (p<0.05) between Th1 and Th2 differentiation as determined by ANOVA followed by post-hoc analysis. Modified and reprinted from *Journal of Immunological Methods* vol. 278, pp. 157-169, 2003, with permission from Elsevier.

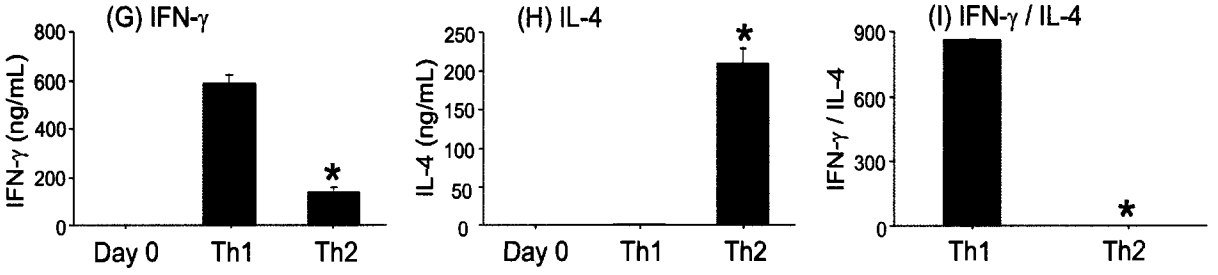
Transcription Factor Gene Expression



Cytokine Gene Expression



Cytokine Protein Secretion



CHAPTER 1 – DISCUSSION

When non-activated naïve T cells encounter their cognate antigen and receive co-stimulatory signals, they can differentiate into Th1 or into Th2 effector cells that secrete different functional cytokines. Cytokines can act in an autocrine manner and are also secreted by other immune cells. The strongest factor regulating CD4 T cell differentiation is the cytokine mixture present in the microenvironment of the cell during activation (17).

The T-bet and GATA-3 transcription factors are the major cell signaling regulators of Th1 and Th2 cell development (6). Studies using purified mouse cell populations suggest that the presence of T-bet or GATA-3 determines the differentiation of naïve CD4 T cells (6). In the present study (Figures. 1-2, 1-3, 1-4, and 1-5), changes in the ratio of expression of T-bet and GATA-3 reflected changes in Th1 and Th2 cytokine expression in mixed populations of control rat spleen cells. The relative expression of T-bet and GATA-3 was more representative of Th1/Th2 cytokine balance than expression of either transcription factor alone (104).

PMA+ionomycin cell stimulation nonspecifically activates gene expression over a 24h period. The Th1 and Th2 stimulation protocol induced a controlled activation that occurred over two weeks. As we described in Chakir, *et al.* (104), PMA+ionomycin stimulation induces IFN-γ production that is STAT-4-independent, while the Th1 stimulation protocol induces STAT-4-dependent IFN-γ expression (128). Therefore, the ratio of T-bet/GATA-3 was a biologically meaningful measure of Th1/Th2 cytokine status with the stimulation protocol which specifically induces these cytokines, but not with PMA+ionomycin cell stimulation. Thus, it is important to first validate the use of this ratio for each model in which it is used.

T-bet upregulation was observed during the first week of Th1 stimulation in rat spleen cells (Fig. 1-1A). High expression was maintained in Th1 cells until as late as day 12 (during the second stimulation; data not shown), but gene expression was down-regulated by day 15 (during the third stimulation; Fig. 1-5A). Despite low T-bet in polarized Th1 cells, IFN-γ expression was maintained (Fig. 1-5D,G), confirming the Th1 phenotype.

GATA-3 gene expression was maintained in long-term polarized rat Th2 cells. It was completely down-regulated in Th1 cells, suggesting that its expression is a better indicator of long-term polarization than expression of T-bet, for both Th1 and Th2 conditions. This indicates that GATA-3, and not T-bet, is the major regulator of the maintenance of Th1/Th2 cytokine status. GATA-3 is one of the major transcription factors which induce Th2 differentiation and maintenance (124, 125). It also inhibits Th1 development by inhibition of IL-12Rβ2 chain expression (125) and possibly by inhibiting the IFN-γ promoter (129). The present results suggest that downregulated GATA-3 expression in Th1 cells is more important than upregulated T-bet expression for the production of IFN-γ.

Th1 or Th2 immune responses are usually identified by the cytokine secretion profile of representative Th1 and Th2 cytokines. In comparison, T-bet and GATA-3 measurement in freshly isolated tissue provides a general reflection of the *in vivo* cytokine status. This procedure also avoids cell isolation procedures which can artificially change gene expression levels.

There is a complex array of cytokines secreted by Th1 and Th2 cells, and IFN-γ and IL-4 can be secreted by cells other than CD4 T cells (Table 1-1). Therefore, cytokine expression does not necessarily reflect only Th1/Th2 cell status. T-bet and GATA-3 expression represents a marker for several cell types which can produce Th1 and Th2

cytokines. Transcriptional changes in these genes occurred very early compared with cytokines during T cell differentiation (Fig. 1-2). The T-bet/GATA-3 ratio could be used to monitor the early development of and the progression of cytokine-mediated autoimmune diseases. It is, however, important to verify the validity of this method for each disease model in which it is used. For example, this method would not detect a newly discovered T-bet-independent effector CD4 T cell subset termed the Th17 lineage. These cells are involved in the pathogenesis of several autoimmune diseases classically thought to be mediated by Th1 mechanisms (130).

The validity of the T-bet/GATA-3 ratio concept has been confirmed in other animal models (131-133). DO11.10 mice are transgenic for a TCR which recognizes a chicken ovalbumin (OVA) peptide. These mice are also deficient in the recombinase-activating gene 2 (Rag2), thus B and T cell receptor genes are not rearranged. In the absence of the cognate OVA antigen, the T cells in these animals remain naïve. In a paper on which I am a co-author (104), we used naive $CD4^+$ splenic T cells from DO11.10/Rag2 $^{-/-}$ OVA TCR-transgenic mice to analyze T-bet and GATA-3 correlation with Th1/Th2 cell development. In support of the present data obtained with BBc rats, Th1/Th2 cytokine gene and protein profiles showed a close correlation with the T-bet/GATA-3 gene expression ratio.

Thus, the T-bet/GATA-3 ratio provided a useful surrogate marker of Th1/Th2 profile that was more consistent than the expression of either transcription factor alone. Changes in GATA-3 expression were the stronger determinant of Th1/Th2 cytokine balance in mixed cell populations (104). This method has since been utilized and confirmed in several publications by other labs including the following references (131-133). The approach of measuring transcription factor expression to determine the overall inflammatory status of immune cells in a tissue provided a useful tool for the characterization of the inflammatory status of the gut of diabetes-prone BioBreeding rats (126).

CHAPTER 2 – Wheat Protein-induced Inflammatory T helper 1 Cells in the Gut and Associated Lymphoid Tissues of Diabetes-prone BioBreeding Rats

CHAPTER 2 – INTRODUCTION

Type 1 diabetes results from inflammatory autoimmune destruction of pancreatic beta-cells. Th1 cells have been implicated in this process (58). Environmental factors can modify disease expression in individuals with genetic risk (58, 60). The origin of beta-cell specific lymphocytes and the identity of the stimulatory antigens remain unknown. Diabetes incidence is strongly influenced by diet in genetically susceptible rodents (38, 57, 58, 60). Incidence in BBdp rats fed wheat gluten protein (WP)-based cereal (CER) diets, such as NTP-2000 (102), is 60-70 % (57). Diabetes incidence is reduced to approximately 20 % in rats fed a wheat-free hydrolyzed casein (HC)-based diet (57). This suggests that wheat proteins have a major effect on the development of diabetes in BBdp rats.

Dietary antigens are first encountered by the gut immune system. Antigens sampled by Peyer's patches (PP) in the gut are presented to lymphocytes by APCs (101). Activated lymphocytes proliferate and traffic through lymphatics to the mesenteric lymph nodes (MLN; 79). Mature lymphocytes can then migrate back to the gut. MLNs are the major inductive site of the gut-associated lymphoid tissue (GALT) where T cells respond to dietary antigens (79). BBdp rats are lymphopenic with low numbers of $CD4^+$ T cells and $CD8^+$ T cells (109), and a reduced frequency of regulatory T cells expressing an *RT6* rat cell surface marker (35). Although not a normal feature of diabetes in humans or NOD mice, mild lymphopenia has been reported in some T1D patients (134, 135), and has been proposed to promote autoimmunity (136).

The gut of BBdp rats displays a mild inflammatory enteropathy, and permeability via the paracellular route (84, 88, 89, 91, 137). Very little is known about the immune mechanisms of this damage and about the immune state of the gut-associated lymphoid tissue (GALT) in diabetes-prone BB rats. It was hypothesized that the gut and GALT of diabetes-prone rats could be a reservoir of inflammatory Th1 lymphocytes that can proliferate in response to antigens in a diabetes-promoting, cereal-based diet.

CHAPTER 2 – EXPERIMENTAL DESIGN

Studies (Table 2-1) were performed at ages that pre-date classic insulitis. First, gut-associated lymphoid tissues from NTP-2000 cereal (CER)-fed BBc and BBdp rats, aged 30 days, were analyzed for gene expression of T-bet and GATA-3 transcription factors. In another experiment, BBc and BBdp rats were fed either a diabetes-retardant HC or a diabetes-promoting CER-based diet to age 45 days to allow full Th1 cell polarization (104), and then duodenum was analyzed for expression of the Th1 cytokine IFN-γ using RT-PCR. In a final experiment, T cells specific for wheat were analyzed in the MLN of BBc and BBdp rats at around age 60 days, the age at which cereal-fed BBdp rats begin to develop diabetes.[b]

[b] The following sections contain some information which is included in the following publications on which I am a co-author: (109) 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. (138) Lefebvre, D.E., H. Chakir, and F.W. Scott. 2004. Wheat gluten-induced pro-inflammatory Th1 bias in mesenteric lymph nodes of diabetes-prone BB rat (Abstract). *FASEB J* 18:A496.

Table 2-1. Experimental groups studied in chapter 2
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	Experiment 1: T-bet / GATA-3 expression ratio		Experiment 2: IFN-γ cytokine expression				Experiment 3: wheat protein-specific T cell phenotype	
Animal	BBc	BBdp	BBc	BBc	BBdp	BBdp	BBc	BBdp
Number of rats per group	5	5	5	5	10	10	6	5-6
Diet	CER	CER	HC	CER	HC	CER	CER	CER
Mean age at kill	30 days		45 days				60 days	

CHAPTER 2 – RESULTS

Increased T-bet/GATA-3 Ratio in the Gut-associated Lymphoid Tissues of Young BBdp Rats

The wall of the gut of some BBdp rats appeared much thicker, opaque, and sporadically inflamed and had a rubbery texture compared with control BB (BBc) rats. Thus, in some BBdp rats, damage to the gut was discernable even to the unaided eye. To obtain a measure of the overall immune inflammatory status of the gut, Peyer's patches (PP), a sampling site of lumen antigens, and MLN, the major inductive site for immune cells draining from the gut, were studied and compared with the spleen, which was used to reflect the systemic immune status. The T-bet/GATA-3 expression ratio in these tissues of BBc and BBdp rats fed a CER-based diet was analyzed as a measure of the Th1/Th2 microenvironment using the techniques described in Chapter 1. At 30 days of age, which is more than five weeks before BBdp rats begin to develop diabetes, the PP and MLN of BBdp rats had an increased T-bet/GATA-3 ratio compared with the tissues of BBc rats (Fig. 2-1). This indicated an early Th1 bias in the gut. The increased T-bet/GATA-3 ratio was mainly due to down-regulated GATA-3 expression and not to increased expression of T-bet (Fig. 2-

1B,E). The T-bet/GATA-3 ratio was not different in the PP or MLN at 23 days, which was the first day after weaning (data not shown). The T-bet/GATA-3 ratio was not increased in spleen of BBdp rats, suggesting that the altered Th1/Th2 balance is specific to the gut and associated lymphoid tissues (data not shown).

Increased IFN-γ Expression in the Duodenum of Cereal-fed BBdp Rats

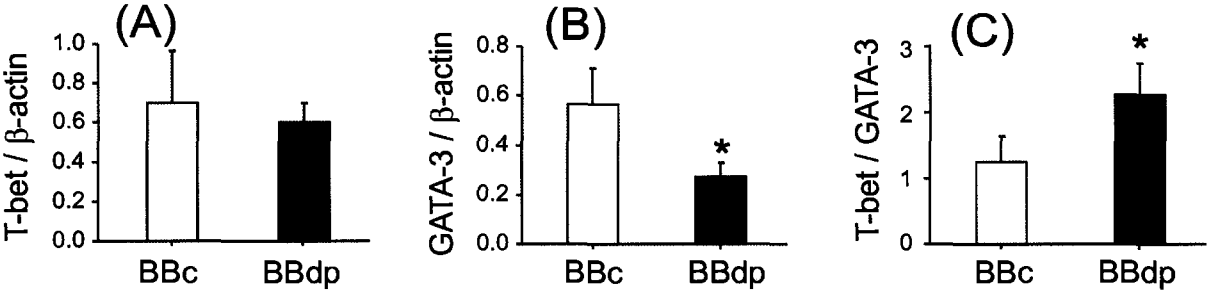
The next experiment was designed to determine whether the early T-bet/GATA-3 imbalance in the inductive BBdp PP and MLN could promote an increased cytokine response to a cereal-based diet. IFN-γ (Th1), IL-10 and TGF-β (Th2/Th3) gene expression ratio was determined at the whole tissue level of BBc and BBdp rats fed a diabetes-promoting CER or a diabetes-retardant HC diet (Fig. 2-2). IFN-γ message was not detectable in the duodenum of 10, 23 or 30 day BBdp rats (data not shown). Expression was detectable at 45 days and was significantly higher in the duodenum of CER-fed compared with HC-fed BBdp rats (2-2A). In BBc rats, IFN-γ expression was not modified by diet. IL-10 message was not different between strains and was not modified by diet (Fig. 2-2B). TGF-β message was reduced only in HC-fed BBdp compared with BBc rats (Fig. 2-2D).

Although IFN-γ was significantly lower in the gut of BBdp compared with BBc rats (Fig. 2-2A) this was likely due to lymphopenia in BBdp rats (109). Lymphopenia results in a lower proportion of T cells to total cells in the tissue. To correct for this, the ratios of IFN-γ to IL-10 and IFN-γ to TGF-β were taken. This calculation removed the difference between BBdp and BBc rats for the diabetes-promoting CER diet (Fig. 2-2C,E).

Figure 2-1. Increased T-bet/GATA-3 ratio in gut associated lymphoid tissues of cereal-fed BBdp rats

Gene expression of T-bet (A, D), GATA-3 (B, E) and T-bet/GATA-3 ratio (C, F) in 30-day-old CER-fed BBc (white bars, n=5) and BBdp rat (black bars, n=5) MLN and PP immediately upon excision of the tissues. Semi-quantitative RT-PCR was performed with triplicate samples from each rat. Data were normalized to β-actin gene expression and represent mean±SD for five rats/group. * indicates a statistically significant difference (p<0.05) between BBc and BBdp rats as determined by ANOVA followed by LSD post-hoc analysis. Modified and reprinted from *Diabetologia* vol. 48, pp. 1576-1584, 2005, with kind permission from Springer Science and Business Media.

Mesenteric Lymph Node



Peyer's Patch

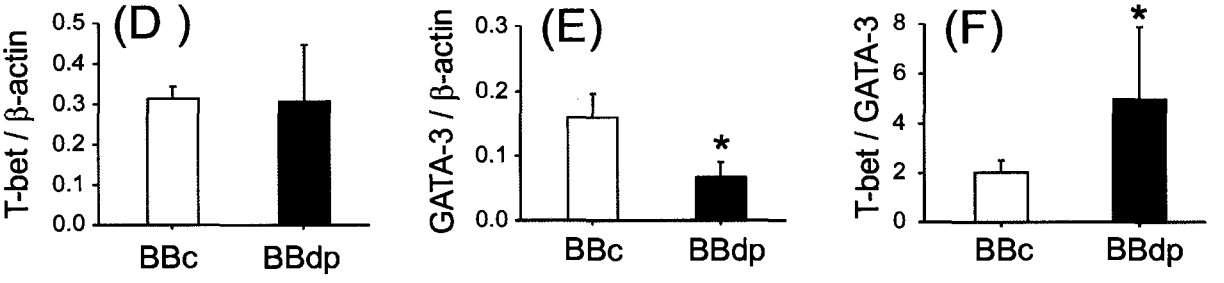
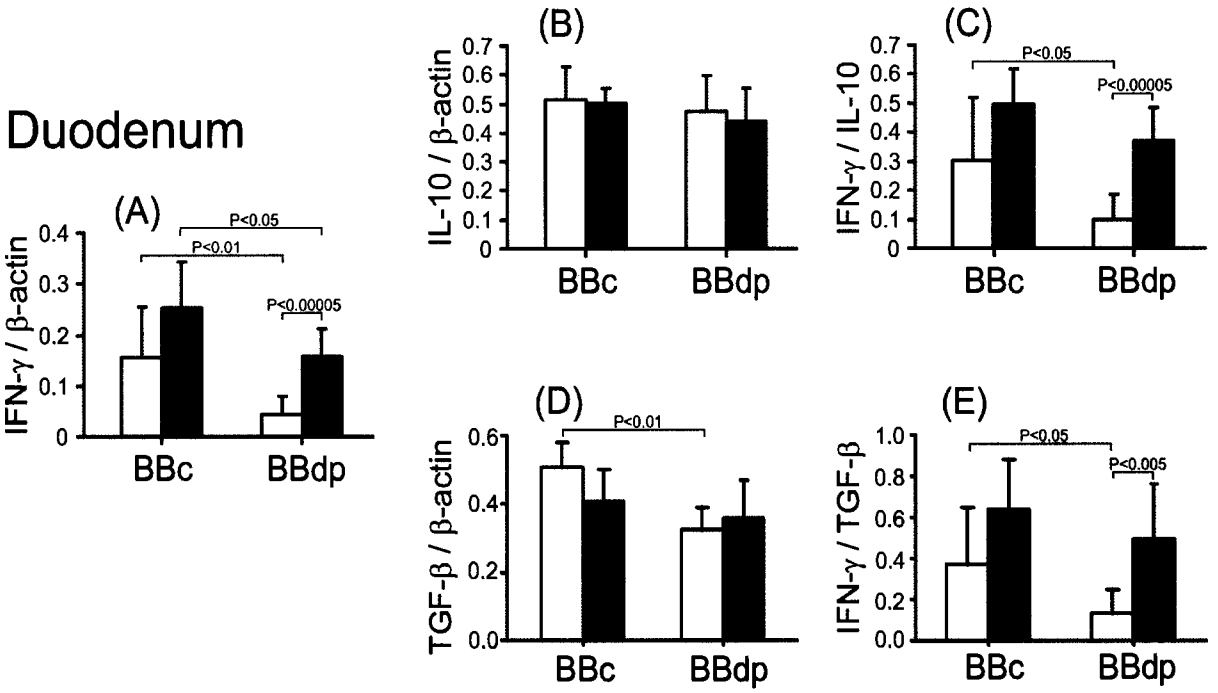


Figure 2-2. Th1 cytokine expression induced by a diabetes-promoting cereal-based diet in the duodenum of BBdp rats

Gene expression of IFN-γ (A), IL-10 (B), IFN-γ/IL-10 ratio (C), TGF-β (D), and IFN-γ/TGF-β ratio (E) was measured in 45 day BBc and BBdp rat duodenum, from which Peyer's patches were removed, immediately upon excision of the tissue. Rats had been fed a diabetes-retardant hydrolyzed casein (HC, white bars; n = 5 BBc, 10 BBdp) or a diabetes-promoting cereal diet (CER, black bars ; n = 5 BBc, 10 BBdp). Semi-quantitative RT-PCR was performed with triplicate samples from each rat. Data were normalized to β-actin expression and represent mean±SD for five BBc and ten BBdp rats. Statistically significant differences between BBc and BBdp rats were determined by ANOVA followed by LSD post-hoc analysis.


Duodenum
(A)
IFN-γ / β-actin
P<0.05
P<0.01
P<0.00005
BBc
BBdp
(B)
IL-10 / β-actin
BBc
BBdp
(C)
IFN-γ / IL-10
P<0.05
P<0.00005
BBc
BBdp
(D)
TGF-β / β-actin
P<0.01
BBc
BBdp
(E)
IFN-γ / TGF-β
P<0.05
P<0.005
BBc
BBdp

Reactivity of T Cells from the MLN of Cereal-fed BBdp Rats to Wheat Proteins

Gut inflammation in BBdp rats is sporadic and could vary along the length of the gut. Therefore the draining lymph nodes (MLN) are a better reflection of overall inflammatory status in the gut (79). Thus, the MLN were subsequently used to identify inflammatory immune cell types. An *in vitro* system was optimized for detection of CD4 T cell responses to antigens with single cell suspensions prepared from the MLN of a control rat. The response was quantified by a CFSE-based flow cytometric proliferation assay. CFSE is a fluorescent label which remains covalently bound to the cell. When CFSE-labeled cells complete mitosis, daughter cells lose half of the dye after each division, resulting in a gradual loss of the CFSE from the proliferating cells, which become $CFSE^{low}$. By contrast, non-dividing cells maintain similar concentrations of CFSE and remain $CFSE^{+}$ after long term culture. This method successfully identified CD4 T cell proliferation in response to anti-CD3 (TCR stimulation) and anti-CD28 (co-stimulatory signal) treatment compared with no proliferation by cells in medium alone (Fig. 2-3). CD8 T cells also responded to anti-CD3 and anti-CD28, but were not quantified.

To further clarify the origin and the nature of the pro-inflammatory cells in the gut and GALT of BBdp rats, the reactivity of immune cells in the MLN to wheat gluten proteins (WP) was examined. To determine whether wheat-reactive CD4 T cells were present at 60 days, just before BBdp rats begin to develop diabetes, the proliferation of MLN cells was evaluated in response to wheat protein *in vitro* (Fig. 2-4). MLN and spleen cells from 60 day BBc and BBdp rats, that had been fed the cereal-based diet for more than 5 weeks, were stained with CFSE and incubated with three concentrations of α-chymotrypsin-treated wheat protein (109). As negative controls, cells were cultured in medium alone or in the presence

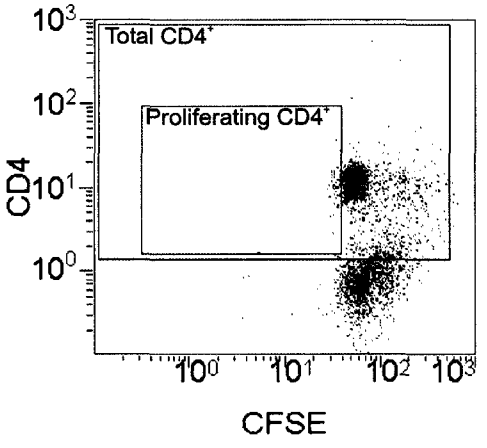
of chicken OVA protein, to which they had never been exposed. As a positive control, cells were incubated with anti-CD3 and anti-CD28 antibodies. Minimal mitosis was detected with exposure to medium alone or to OVA protein. MLN T cells from both BBc and BBdp rats proliferated following activation by anti-CD3 and anti-CD28 treatment. $CD4^+$ T cells from MLN of BBdp rats proliferated in a dose-dependent manner to different concentrations of wheat protein (Fig. 2-4). $CD4^-$ MLN cells, which consist mainly of B cells, dendritic cells, and some $CD8^+$ T cells, showed little response in BBdp rats (data not shown). CD4 T cells in the MLN from BBdp rats had significantly greater response to wheat protein compared with control rats. This cellular proliferation was greater in the MLN than in the spleen (Fig. 2-4). In rats, CD4 is expressed not only at high levels on T lymphocytes but also at low levels on monocytes and macrophages. The identity of the cells which responded to wheat was clarified by flow cytometry with cells co-stained with antibodies which detect CD4 and antibodies specific for αβ-TCR, the major T cell receptor isotype. After gating on wheat protein-reactive $CD4^+$ cells, approximately 95 % of the cells were confirmed to be αβ-TCR-expressing T cells (Fig. 2-4C).

To determine why wheat-specific T cells proliferate in diabetes-prone, but not control rats, flow cytometry was used to analyze freshly isolated MLN and spleen cell single cell suspensions from 60-day-old CER-fed BBc and BBdp rats. Compared with those from BBc animals, the MLN and spleen from BBdp rats contained a lower proportion of cells with a $CD4^+CD25^+$ T cell phenotype (Fig. 2-5).

Figure 2-3. Proof of principle: CFSE staining to monitor cellular proliferation *in vitro*

Cells from the MLN of a 60 d cereal-fed BBc rat were stained with fluorescent CFSE label. Cells were then plated in medium only or with anti-CD3 and anti-CD28 antibodies. At d 3, cells were stained with anti-rat CD4-cychrome antibody and analyzed for cell proliferation by flow cytometry. Gates were used to measure the ratio of proliferating $CD4^+$ cells (dilution of the CFSE label after each division) to total $CD4^+$ cells. Data are representative of six experiments.

Medium



anti-CD3 + anti-CD28

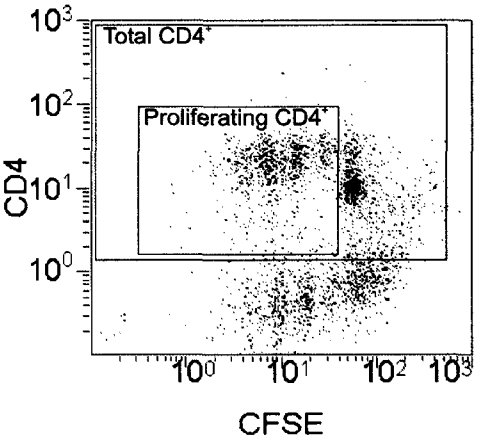
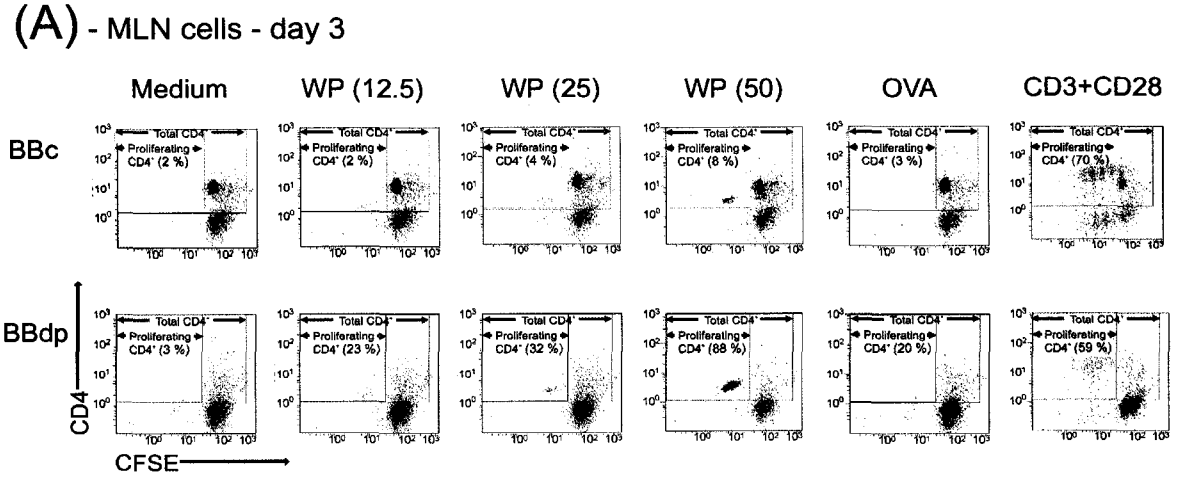
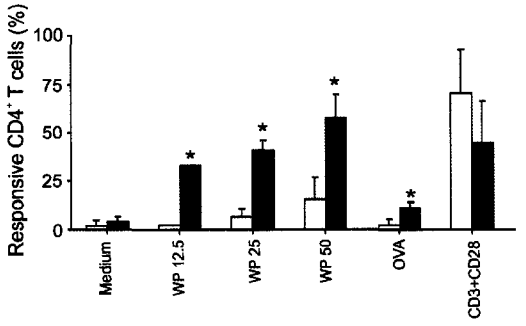
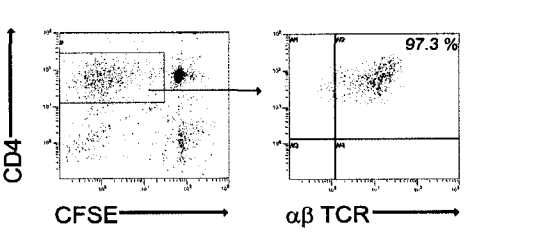


Figure 2-4. *Ex vivo* response of BBdp rat MLN and spleen cells

Cells from the MLN (A-C) or spleen (D) of 60 d CER-fed BBc rats (white bars, n=6) and BBdp rats (black bars, n=6) were stained with CFSE and cultured in the presence of three concentrations of wheat proteins (WP, μg/ml). Control cultures contained medium only or OVA protein (negative controls), or anti-CD3 and anti-CD28 antibodies (positive control). At d 3, cells were stained with cy-chrome-conjugated anti-rat CD4 and cell proliferation was analyzed by flow cytometry. (A) Representative flow cytometry results from one BBc and one BBdp rat. (B, D) Mean±SD of the ratio of proliferating $CD4^+$ cells to total $CD4^+$ cells for six rats/group. * indicates a statistically significant difference, (p<0.05) between BBc and BBdp rats as determined by ANOVA followed by LSD post-hoc analysis. (C) BBdp MLN cells were cultured for 7 days in the presence of 50 μg/ml wheat protein and then stained with cy-chrome-conjugated anti-CD4 and PE-conjugated anti-αβ-TCR antibodies. $CD4^+$ MLN cells proliferating to wheat protein antigens ($CD4^+$CFDA-SE^{low}) were gated (open box) and analyzed by flow cytometry to assess the proportion of αβ-TCR^+ cells. Modified and reprinted from *Diabetologia* vol. 48, pp. 1576-1584, 2005, with kind permission from Springer Science and Business Media.


(A) - MLN cells - day 3
Medium
WP (12.5)
WP (25)
WP (50)
OVA
CD3+CD28
BBc
BBdp
Total CD4+
Proliferating CD4+ (2 %)
Proliferating CD4+ (2 %)
Proliferating CD4+ (4 %)
Proliferating CD4+ (8 %)
Proliferating CD4+ (3 %)
Proliferating CD4+ (70 %)
Proliferating CD4+ (3 %)
Proliferating CD4+ (23 %)
Proliferating CD4+ (32 %)
Proliferating CD4+ (88 %)
Proliferating CD4+ (20 %)
Proliferating CD4+ (59 %)
CD4
CFSE


(B) - MLN cells - day 3
Responsive CD4+ T cells (%)
100
75
50
25
0
Medium
WP 12.5
WP 25
WP 50
OVA
CD3+CD28


(C) - BBdp MLN cells - day 7 - WP 50
97.3 %
CD4
CFSE
αβ TCR

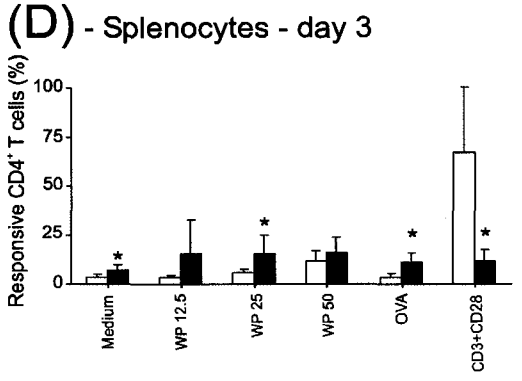
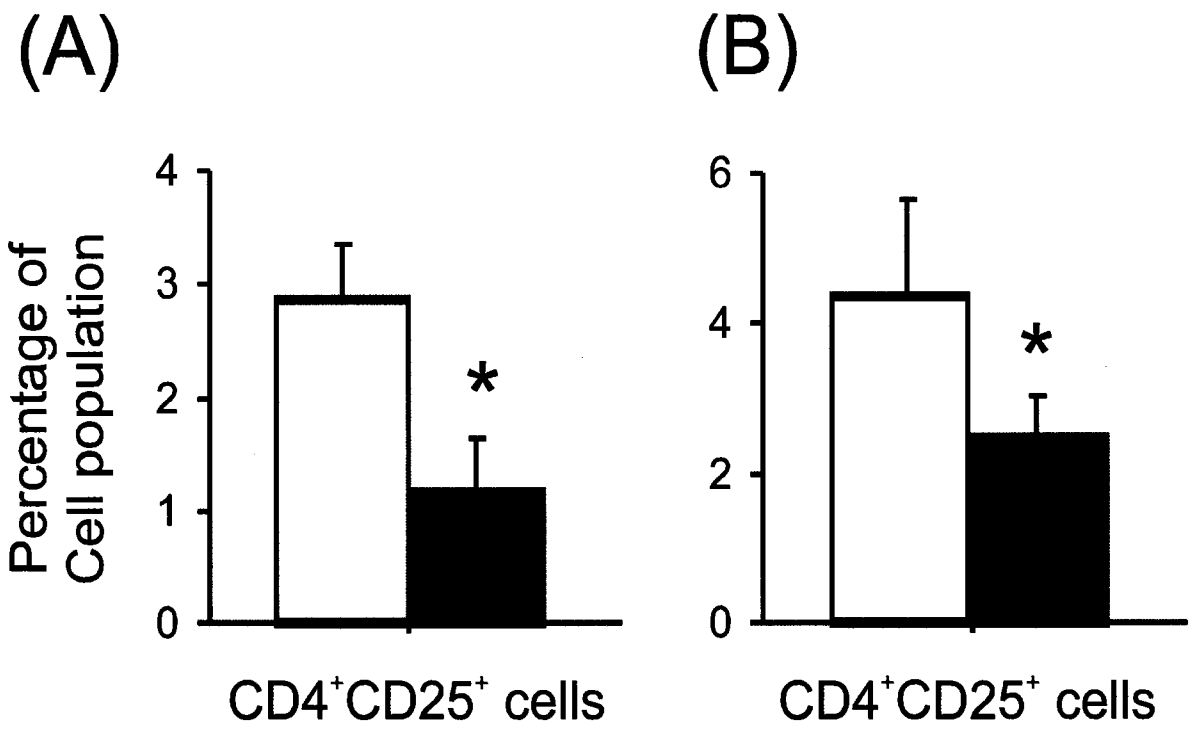

(D) - Splenocytes - day 3
Responsive CD4+ T cells (%)
100
75
50
25
0
Medium
WP 12.5
WP 25
WP 50
OVA
CD3+CD28

Figure 2-5. $CD4^+CD25^+$ T cells in non-stimulated BBdp rat MLN and spleen cells

Freshly isolated resting MLN cells (A) or spleen cells (B) from 60-day CER-fed BBc rats (white bars, n=6) and BBdp rats (black bars, n=5) were co-stained with antibodies specific for activated or regulatory T cell surface markers (CD4 and CD25) and the percentage of cells expressing these markers was analyzed by flow cytometry gated with dot plots gated on lymphocytes. Data represent the means±SD of the percentage cell population. * indicates a statistically significant difference in cell populations (p<0.05) between BBc and BBdp rats as determined by ANOVA followed by LSD post-hoc analysis. Modified and reprinted from *Diabetologia* vol. 48, pp. 1576-1584, 2005, with kind permission from Springer Science and Business Media.


(A)
(B)
Percentage of Cell population
4
3
2
1
0
6
4
2
0
*
*
CD4+CD25+ cells
CD4+CD25+ cells

CHAPTER 2 – DISCUSSION

As discussed in a paper on which I share first authorship (109), the inflammation that destroys islet beta-cells in the pancreas of BBdp rats includes a Th1 component, which is characterized by the production of IFN-γ (38-41). However, the origin of the cells which produce these cytokines, the antigens which stimulate these cells, and the age at which they become activated are not known. The gut has been proposed as a source of these cells (56, 139, 140), but the inflammatory condition of the gut, the phenotype of the cells, and the involvement of stimulatory dietary antigens in the period before diabetes development are not known.

The gut mucosal epithelial cell barrier controls the entry of antigens and commensal bacteria. In healthy individuals, exposure to low levels of sampled dietary antigens leads to oral tolerance (79). Tolerized immune cells in the gut and GALT maintain Th2 cytokine production. The present study shows that such an immunosuppressive Th2 environment was not maintained, particularly in the inductive MLN tissue, in young cereal-fed BBdp rats.

Th1/Th2 differentiation is influenced by the relative expression of Th1-specific T-bet and Th2-specific GATA-3 (Fig. 1-5). T-bet and GATA-3 expression in fresh BBdp MLN and PP tissues revealed a decrease in GATA-3 expression along with no change in T-bet expression. Thus, the GALT of BBdp rats displays a Th1 bias that is the result of a relative Th2 deficit. This Th1-promoting status developed 1 week after weaning (Fig. 2-1), long before islet inflammation develops, and was only present after weaning to a cereal-based diet. This suggests that the introduction of dietary components could be an important factor promoting Th1 development. There was no change in T-bet/GATA-3 ratio in the spleen

(data not shown) suggesting a different Th1/Th2 balance in the systemic compared with the gut immune system.

Effector and memory cells which mature in the MLN can traffic through the circulatory system back to the gut mucosa (141). To measure whether differentiated Th1 cells, as developed in the MLN at 30 d (Fig. 2-1), were also detectable in the gut mucosa, whole duodenum tissue was analyzed for IFN-γ expression. This analysis was performed with 45-day-old rats to allow 3 weeks of exposure to dietary antigens and to ensure full Th1 polarization of naïve T cells as described in Chapter 1 (Fig. 1-5). A marked dietary effect was observed in BBdp rats. CER-fed BBdp rats developed a Th1 cytokine bias in the duodenum, compared with HC-fed animals, which was characterized by an increased IFN-γ/IL-10 ratio. Strain differences were not as clear. The increased T-bet/GATA-3 in the inductive PP and MLN of BBdp rats at day 30 (Fig. 2-1) was not reflected by differences in cytokine expression between BBc and BBdp duodenum at day 45 (Fig. 2-2). Determination of the gene expression status of a specific cell population in a whole tissue can be confounded if lymphopenia is present because expression is normalized to β-actin, which is expressed in both the target and in other cells. The effect of lymphopenia was corrected by taking the ratio of IFN-γ to IL-10 and to TGF-β cytokines. Following this, there were no major differences in cytokine expression between BBc and BBdp rats. The contrast with the increased T-bet/GATA-3 ratio in the BBdp MLN is likely related to the comparison of duodenum with the inductive MLN from groups with different ages, and due to the sporadic nature of damage along the gut. The increased T-bet/GATA-3 ratio in the BBdp MLN did reflect IFN-γ levels in the same tissue when detected with intracellular flow cytometry gated on CD4 T cells (109).

In the paper on which I share first authorship (109), intracellular flow cytometry was used to quantify IFN-γ producing $CD4^+$ T cells in the MLN of BBc and BBdp rats fed HC or CER. By first gating on $CD3^+$ T cells, the skewing effect of BBdp rat T cell lymphopenia was removed. This allowed for specific comparison of IFN-γ expression in the $CD4^+$ T cell population. The MLN of BBdp rats, had a high proportion of $CD3^+CD4^+$ IFN-γ^+ Th1 cells compared with BBc rats. Consistent with the gene expression data (Fig. 2-2), these cells were reduced to control levels in BBdp rats fed an HC diet. IFN-γ-expressing Th1 cells in the spleen were not altered by diet, which suggests that the dietary activation of these Th1 cells is gut specific. Taken together, these data confirm that the *in vivo* profile of activated BBdp gut mucosal T cells favours the Th1 pathway. They also suggest that Th1 cytokine expression was suppressed by an HC diet in the gut of BBdp rats. This is consistent with the report that mitotic activity is high in BBdp MLN and is blocked in HC-fed rats (92, 142).

Increased gut permeability is present in BBdp rats (84, 85, 92). This leaky gut mucosal barrier could allow abnormal exposure to dietary protein antigens in the mainly wheat-based cereal diet. To determine if the Th1 bias in the gut and MLN of cereal-fed BBdp rats (Fig. 2-1)(109) was related to the activation of wheat protein-specific immune cells, CFSE labeling and flow cytometry (110) were used to identify cells which respond to wheat in the MLN of rats fed a wheat containing diet for 5 weeks. Gating on $CD4^+$ T cells and then determining the ratio of proliferating T cells corrected for the effect of lymphopenia. This method allowed the identification of wheat protein-specific $CD4^+$ T cells in the MLN of BBdp rats (Fig. 2-4B). BBdp spleen cells did not react to wheat, suggesting that they are predominantly present in the gut. This could also be related to the increased frequency of inhibitory macrophages in the spleen of BBdp rats (143). The abnormal

activation of wheat protein-specific $CD4^+$ T cells in the MLN of BBdp rats could occur due to the sporadic inflammatory state of the gut (89) and to the low frequency of $CD4^+CD25^+$ T cells (Fig. 2-5), a population which contains regulatory T cells (44). It could also be related to increased antigen-presenting cells, which increase the potential for presentation of dietary antigens to T cells (109). Wheat proteins can also induce the maturation of dendritic cells (144). The strong reaction of CD4 and not CD8 T cells (Fig. 2-4A) implies that WP antigens are presented mainly by MHC class II molecules to CD4 T cells. Alternatively, the lack of a CD8 response could be related to the severe CD8 T cell lymphopenia (109) or to unfavorable *in vitro* culture conditions.

It was previously shown that young BBdp rats have a damaged gut (89) and increased permeability (84, 85, 92). It is tempting to speculate that the high proportion of IFN-γ-expressing T cells in the BBdp gut induce this histological damage, similar to the case in patients with the wheat-induced gut disorder celiac disease (145, 146). However, while Th1 cytokines in the BBdp gut were modified by dietary wheat (Fig. 2-2; 109), histological gut damage was not (89). Despite this, a wheat-based diet did modify gut permeability and other signs of functional gut damage (85, 92, 147). A recent study of patients with T1D and various degrees of celiac disease identified IFN-γ and other inflammatory markers in the jejunal mucosa (90). The pro-inflammatory state in the GALT of BBdp rats is consistent with these findings.

Similar to the dietary effect on diabetes incidence (57; Fig. 5-2), gut permeability is also reported to be modified by diet (85, 88) and has been linked to diabetes outcome (87). The present work identifies wheat-specific activation of Th1 cells in the gut and MLN of BBdp rats. CD4 T cells play a role in diabetes development in BBdp rats (148). Low levels

of cytotoxic CD8 T cells are also required (Fig. 2-4A; 42). The Th1 cytokine-producing cells activated in the BBdp gut (89) could play a role in the dietary modification of type 1 diabetes. Studies with NOD mice have shown that adoptively transferred MLN cells can induce diabetes (98), and lymphocytes from the MLN have been shown to traffic to the pancreas (149). However the role of wheat-reactive T cells in diabetes pathogenesis has not yet been determined.

Thus at an age when islet damage is absent, there was a wheat-induced Th1 cytokine bias in the GALT of BBdp rats, as reflected by an increased T-bet/GATA-3 ratio and high IFN-γ expression. This Th1 bias was related mainly to decreased GATA-3 expression, supporting the concept that GATA-3 is the major regulator of Th1/Th2 balance. Despite lymphopenia, the MLN cells of cereal-fed BBdp rats proliferated strongly in a specific dose-dependent manner to wheat antigens (138). This process could result from the high expression of inflammatory Th1 cytokines in a microenvironment which lacks T regulatory cells (44). These results raised the question of whether a similar immune response to wheat proteins is present in patients with T1D.

CHAPTER 3 – Wheat Protein-Reactive T cells in a Subset of Patients with Type 1 Diabetes

CHAPTER 3 – INTRODUCTION

Early exposure to dietary wheat was associated with islet autoimmunity in infants with genetic risk (75, 76). However the role of wheat protein in diabetes pathogenesis is unknown. As many as 16 % of type 1 diabetes patients also have the wheat gluten protein-induced enteropathy, celiac disease (CD), compared with 0.4% of the general population (150). The pathogenesis of celiac disease involves T cell activation by gliadin peptides in dietary gluten from grains including wheat (111). This response activates Th1 cells resulting in damage to the gut mucosa (151). T1D and CD have common HLA haplotype risk genes including HLA-DQ2 and HLA-DQ8. As well, children with T1D sometimes have CD-associated autoantibodies to tissue transglutaminase and have antibodies to gliadin (152). It is possible that diabetes-related sporadic immune response to wheat gluten protein (WP) could be present in individuals with diabetes without clinical signs of celiac disease. In T1D patients without celiac disease, there was no gut villus atrophy or inflammation by light microscopy (83). However, transmission electron microscopy revealed changes in tight junctions resulting in increased intercellular spaces, which indicate the presence of a leaky gut barrier. Thus analyzing the cellular immune response to wheat proteins in patients with T1D is highly relevant. It was hypothesized that a sub-population of individuals with type 1 diabetes have abnormal Th1 immune reactivity to wheat proteins.

CHAPTER 3 – EXPERIMENTAL DESIGN

A patient with type 1 diabetes, celiac disease and severe sensitivity to wheat was the index case for a study on immune reactivity to wheat in patients with diabetes. Her peripheral blood mononuclear cells (PBMC) were sampled during clinical remission with respect to celiac symptoms. Control subjects with no significant medical history of autoimmune diseases were also investigated. Proliferation of PBMCs in response to dietary proteins was studied by *in vitro* culture. The Th1/Th2 cytokine profile of these cells was determined in cells stimulated twice with wheat proteins.

Immune reactivity to wheat proteins was also evaluated by CFSE proliferation assay with PBMCs prepared from the blood of 15 patients with T1D (but without clinical signs of CD) and 9 healthy controls (Table 3-1). Wheat-specific T cell lines were generated and used to determine immune cell phenotype, Th1/Th2 cytokine secretion, and T cell specificity for wheat.[c]

Table 3-1. Study of immune reactivity against wheat proteins in patients with T1D
Control subjects and patients with T1D were recruited. Study groups were sex and age matched.

Patients	Number	Female/Male Ratio	Mean Age (years ± SD)	Age Range (years)	Mean Years Since Diabetes Diagnosis (years ± SD)	Range of Years Since Diabetes Diagnosis (years)
Control	9	1.25	25.7 ± 5.8	21-40	-	-
T1D	15	1.5	22.1 ± 7.4	8-38	10.9 ± 5.6	4-19

[c] The following sections contain some information which is included in the following publications on which I am a co-author: (153) 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. (154) Mojibian, M., D.E. Lefebvre, H. Chakir, and F.W. Scott. 2005. T-cell reactivity to wheat proteins in human type 1 diabetes (Abstract). *Diabetes* 54:A79.

CHAPTER 3 – RESULTS

Immune Reactivity to Wheat Protein in PBMCs from a Patient With Type 1 Diabetes and Celiac Disease

A highly wheat-sensitive patient with T1D and CD was enrolled in a study of T cell reactivity to wheat proteins. This 28-year-old Caucasian female, who had T1D for 10 years, presented with diarrhea, oral ulcerations and swelling. At diagnosis, celiac-associated anti-gliadin IgG, anti-tissue transglutaminase IgA, and anti-endomysial antibodies were present.

> "The diarrhea and lip swelling improved initially with a gluten-free diet, but the symptoms recurred within a few months with worsening of the oral ulcerations, lip swelling, and continued diarrhea. Despite 6 months on a standard gluten-free diet, a duodenal biopsy showed mild villous atrophy. ... The patient was evaluated by a clinical allergist. Skin prick tests for several foods, including wheat, were all negative, and there was no evidence of IgE-mediated food allergy" (153).

The patient then experienced inflammation and ulceration of the mucosa of her lower lip and severe diarrhea. She began a strict cereal-free diet (155). Within several weeks the diarrhea and mouth ulcerations resolved and have remained stable. This patient's strong sensitivity to wheat provided an opportunity to characterize the immune response to WP in a diabetic individual with celiac-related gut damage.

I investigated her *in vitro* T cell response to WP using PBMCs isolated during clinical remission and compared with PBMCs from age-matched healthy control subjects (n=3). PBMCs were incubated with wheat gluten proteins (WP) or with control treatments. PBMCs from the patient with T1D and CD proliferated and had an elongated, activated, and clustered appearance by day 14 after culture with WP. PBMCs from control patients did not proliferate in response to WP and did not survive *in vitro*. To generate a polyclonal WP-specific T cell line for RT-PCR analysis of cytokine gene expression, WP-stimulated cells from the patient with T1D and CD were rested with recombinant human IL-7 from day 14-

20, then subjected to another day of stimulation with WP. The IFN-γ/IL-4 gene expression ratio was high, even at baseline, in PBMCs from the patient with T1D and CD and was upregulated in cells stimulated with WP (data not shown).

Immune Reactivity to Wheat Proteins in a Subset of Patients with T1D

To characterize T cell reactivity to wheat in patients with T1D but no clinical signs of celiac disease or autoimmune diseases, PBMCs were isolated, with informed consent, from the blood of patients with T1D and of healthy controls. An *in vitro* CFSE-based flow cytometric proliferation assay was optimized to detect the human T cell response to antigens.

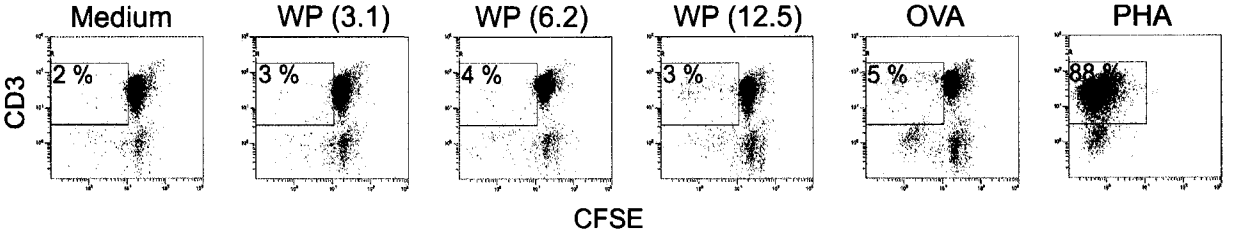
$CD3^+$ T cells in PBMCs from a subset of patients with T1D proliferated when cultured in the presence of WP. In response to 6.2 μg/mL WP, the $CD3^+CFSE^{low}$ accounted for 76 % of the cells in the culture well for the T1D patient exemplified in Fig. 3-1B. The mean proliferation to 12.5 μg/mL wheat protein was significantly increased compared with healthy controls (Fig. 3-1C). Cells from control patients showed minimal response to WP and died by day 7-9 of culture. Patients and control cells had minimal background proliferation in the absence of antigen or in the presence of chicken egg OVA dietary control protein. The response to PHA was similar in T1D patients and controls following three days of *in vitro* culture (data not shown), but was significantly greater in T1D compared with control patients following seven days of culture (Fig. 3-1C).

To test their specificity for wheat, $CD3^+CFSE^{low}$ wheat-reactive T cells from five of the T1D patients which responded to WP were purified by MoFlo cell sorting, rested, and re-stimulated with wheat proteins. Cells proliferated and secreted IFN-γ and low levels of IL-5 specifically in response to wheat during the second stimulation (data not shown). Following the second wheat stimulation, the cells which were present included mostly $CD4^+$ or $CD8^+$ T cells, and small populations of NKT and NK cells (Fig. 3-2).

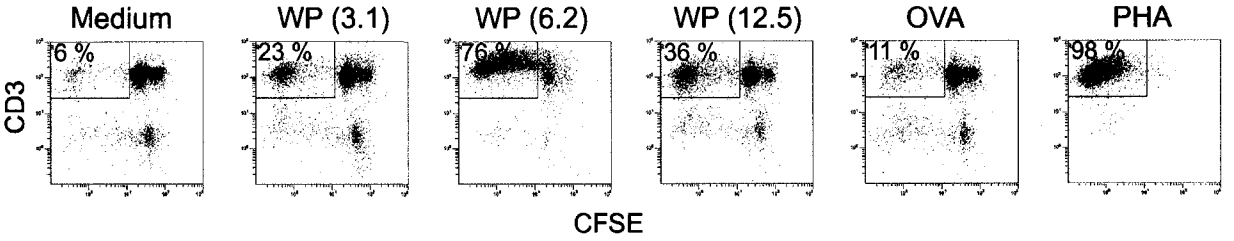
Figure 3-1. T cell reactivity to wheat protein in PBMCs from patients with type 1 diabetes

CFSE-labeled PBMCs from healthy control subjects (n=8) or patients with type 1 diabetes (n=15) were cultured for 7 days in the presence of α-chymotrypsin-treated wheat protein (WP; μg/mL). As controls, cells were cultured in the absence of antigen (Medium), with 44 μg/mL OVA protein or with 2.5 μg/mL PHA. On day 7, cells were stained with sprd-conjugated antibodies specific for human CD3. The percentage of proliferating $CD3^+$ cells was determined by flow cytometry. Representative plots from a control subject (A) and from a patient with T1D (B) are displayed. Gates represent the percentage of T cells which have undergone cell division ($CD3^+CFSE^{low}$). The percentage of proliferating T cells was determined for each patient and bars indicate mean values (C). Statistically significant differences (p<0.05) between controls and T1D patients were determined by ANOVA with followed by LSD post-hoc analysis.

(A) A Control Subject



(B) A Patient with T1D



(C) Mean T Cell Proliferation

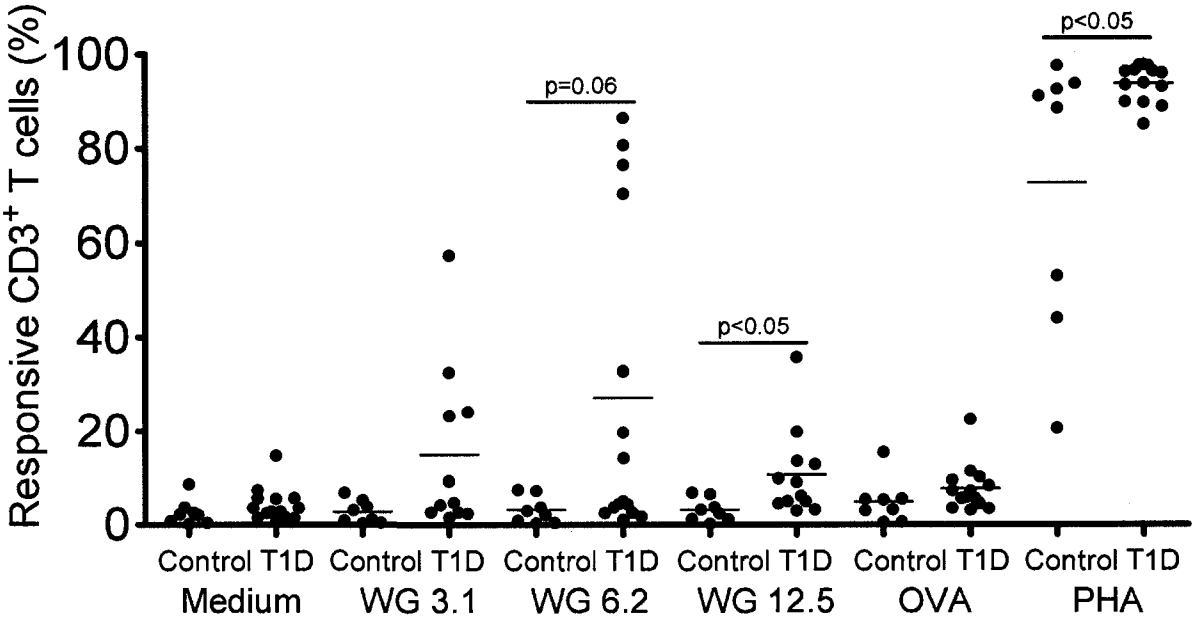
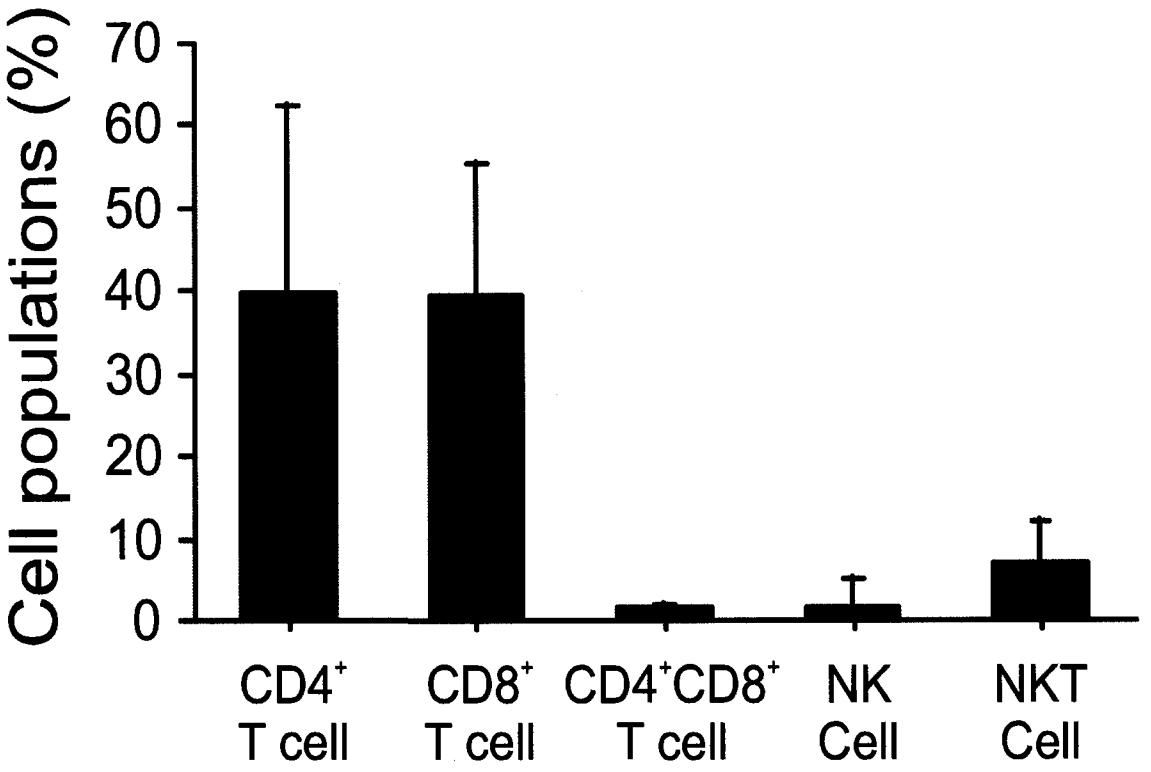


Figure 3-2. Cell surface phenotype of wheat protein-specific T cell lines from patients with T1D

Unsorted, WP-specific T cells from patients with T1D (n=4) were re-stimulated *in vitro* with 6.25 μg/mL α-chymotrypsin-treated WP in RP-15H medium. Irradiated autologous PBMCs were included as antigen presenting cells. Cells proliferated specifically in response to wheat proteins and were harvested after 7-14 d of the second stimulation. Cells were stained with anti-human CD3-sprd, anti-human CD4-FITC, and anti-human CD8-PE to identify $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^+CD4^+CD8^+$ T cells. Another sample of cells was stained with anti-human CD3-sprd and anti-human CD56-PE to identify $CD3^-CD56^+$ NK cells and $CD3^+CD56^+$ NKT cells. Cells were analyzed by flow cytometry. Data represent mean ± SD of the percent cell populations from four patients.


Cell populations (%)
70
60
50
40
30
20
10
0
CD4+ T cell
CD8+ T cell
CD4+CD8+ T cell
NK Cell
NKT Cell

CHAPTER 3 – DISCUSSION

A patient with type 1 diabetes and celiac disease showed unusually high sensitivity to wheat protein. Her sensitivity to wheat was present even when avoiding dietary gluten, which usually decreases immune reactivity to wheat in celiac patients (145). Despite finally adopting a strict cereal-free diet which resolved the symptoms of celiac disease for more than 3 years (155), her *in vitro* immune response to wheat was similar to that of celiac patients recently re-challenged with wheat (145). This response included strong T cell proliferation and a Th1 cytokine-predominant response to a mixture of WP antigens, as indicated by heightened expression of the Th1 cytokine IFN-γ. The persistence of cellular immune reactivity to wheat despite the disappearance of the symptoms suggests that the strict cereal-free diet which the patient initiated was beneficial because it lacks the immunogenic proteins which activate these cells. These results were published in a case report on which I am a co-author (153).

This case report demonstrates that the presentation of wheat peptides to the patient's T cells occurred primarily on HLA-DR (153). Antibodies and T cells specific for the diabetes-related wheat protein Glb1 were also present. Analyses in which I participated showed that PBMCs and WP-specific $CD3^+$ T cells from the patient with T1D and CD proliferated and secreted IFN-γ in response to wheat protein and to the α-gliadin 33-mer wheat peptide, a key celiac-related peptide which is resistant to digestive proteases (data not shown; 111). Immune reactivity to the diabetes-related autoantigens insulin and GAD was also investigated. The patient's PBMCs responded and secreted IFN-γ in response to human insulin and to recombinant GAD. PBMCs from healthy control subjects did not respond.

There is an unusually high frequency of T1D patients with CD, ranging from 0.6 to 16.4 % (156). In coincident cases, 90 % of patients develop T1D first, as was the case in the index patient. There is speculation that the limited number of coincident cases where diabetes is diagnosed second is related to protection from diabetes due to the avoidance of wheat gluten as a treatment for CD (157). The T1D and CD association could also be attributable to shared risk genes (HLA-DQ2/DQ8) or may represent an extreme form of wheat sensitivity in individuals with diabetes. Because the patient with T1D and CD has both diseases, her *in vitro* immune response to wheat proteins cannot be attributed to one disease or the other. Thus, patients with T1D and no clinical signs of CD (tissue transglutaminase antibody negative) were studied to determine if they also display immune reactivity to wheat gluten proteins.

A CFSE-based proliferation assay allowed the detection of immune reactivity of PBMCs from T1D patients to wheat proteins (Fig. 3-1). The low response to a high wheat protein concentration could be related to the toxicity of gliadin proteins in the wheat gluten preparation to cells cultured *in vitro*. The increased T cell response was not related to differences in the proportion of PBMCs expressing the T cell markers (CD3 or β7-integrin), a B cell marker (CD19), or a dendritic cell marker (CD83), which were similar in patients and controls (data not shown). The response to the positive control mitogen, PHA, was greater in T1D patients (Fig. 3-1C). The opposite was true with the T cell activating positive control treatment in diabetes-prone BB rats (Fig. 2-4).

A previous study using a thymidine incorporation assay demonstrated T cell proliferation in response to wheat gluten in T1D patients (158). The present findings (Figs. 3-1, 3-2) confirm these results and used a more sensitive assay to characterize the

proliferating lymphocytes which are present in a significant subset of T1D patients. These data were preliminary and were used as proof of concept to obtain a grant from the Juvenile Diabetes Research Foundation to expand this study. The analysis has continued with many more patients and has confirmed the existence of a subset of T1D patients with reactivity to wheat proteins (154).

Analyses in which I participated confirmed the specificity of this primary response. WP-reactive T cells from five T1D patients were purified by MoFlo cell sorting, and thymidine incorporation proliferation assays were performed upon re-stimulation with wheat proteins in the presence of irradiated APCs. Dose-dependent T cell proliferation was observed in response to WP (data not shown). There was no response to medium or β-lactoglobulin control antigen. Thus the T cell response to wheat was specific and did not occur due to non-specific *in vitro* priming. Unlike the mainly CD4 T cell response to wheat in diabetes-prone BB rats (Fig. 2-4), which are severely lymphopenic with respect to CD8 T cells (109), the T cell lines generated from T1D patients consisted mostly of an equal percentage of CD4 and CD8 T cells (Fig. 3-2). Thus either exogenous wheat antigens presented on MHC class II activate CD4 T cells which then activate CD8 T cells in a bystander manner (159), or wheat antigens are presented on MHC class I to CD8 T cells by cross-presentation (160).

The phenotype of wheat-specific cells from the patient with type 1 diabetes and celiac disease and a subset of patients with T1D was skewed toward Th1 cytokine production as demonstrated by high IFN-γ and low levels or Th2-specific cytokines (data not shown). IFN-γ was suppressed at high WP concentrations in one T1D patient, consistent with the toxic effect of high concentrations of gliadin on cells cultured *in vitro* (161). Insulin and

GAD-specific T cells, which arise during T1D development, were detectable in PBMCs from the patient with T1D and CD and had a Th1 cytokine profile. Thus, the wheat-reactive T cells had a Th1 phenotype similar to that of beta-cell-specific autoimmune T cells.

While not statistically significant, immune reactivity against dietary wheat had an inverse relationship with the number of years since diagnosis (data not shown). This could indicate that the response to wheat subsides following the loss of beta-cells and that this response is temporally linked to the period when T1D develops. However the mechanism by which dietary wheat could be associated with the autoimmune attack on insulin-secreting beta-cells in humans is not known. It is possible that the heightened reactivity to wheat could simply be a secondary effect of diabetes pathogenesis or a disease complication. It is unclear to what extent wheat-reactive cells reflect sub-clinical gut damage and/or the destructive process in the pancreas. However the data suggest that the subpopulation of T1D patients with reactivity to wheat might be at risk for developing overt celiac disease.

Thus results originally obtained in diabetes-prone BB rats were confirmed in a subset of patients with T1D. These include the identification of a population of wheat-specific T cells displaying inflammatory Th1 cytokine expression. Unlike the BB rat, which is severely lymphopenic with respect to CD8 cytotoxic T cell, the response to wheat in humans was mediated by both $CD4^+$ and $CD8^+$ T cells.

CHAPTER 4 – Lack of A Protective Effect of Dietary Hydrolyzed Casein On Type 1 Diabetes in the Komeda Diabetes-Prone Rat, a Cbl-b mutant

CHAPTER 4 – INTRODUCTION

The Komeda diabetes-prone (KDP) rat is a unique model of spontaneous autoimmune T1D which was derived from diabetic Long-Evans Tokushima Lean rats (162, 163). T1D development in the KDP rat resembles human disease pathogenesis in that it occurs at an equal frequency in males and females and in rats which are non-lymphopenic. The KDP rat displays multi-organ inflammation and autoimmunity (114) similar to other models of T1D, however unlike other models, the disease results from a nonsense mutation of the Cbl-b T cell regulator gene. This model also requires the same $RT1^{u}$ MHC haplotype as the BBdp rat for T1D susceptibility (114, 164, 165).

Approximately 80-85 % of diabetes-prone KDP rats fed a standard cereal (CER)-based diet develop diabetes by 210 days of age (113, 114, 165, 166). CER-based diets are also associated with high T1D frequencies in diabetes-prone BB (BBdp) rats and non-obese diabetic (NOD) mice, whereas a hydrolyzed casein (HC) diet significantly reduced T1D incidence in these models (57, 60). The protective effect of the HC diet was associated with increased islet area in the BB rat (38) and reduced insulitis in the NOD mouse (68) upon histological analysis. In the present study, it was hypothesized that the hydrolyzed casein diet which retards T1D in BBdp rats and NOD mice would have a similar effect in KDP rats.

CHAPTER 4 – EXPERIMENTAL DESIGN

Segregating inbred KDP rats were obtained with the assistance of Dr. Susumu Seino and Dr. Norihide Yokoi at Chiba University (Chiba, Japan). Wild-type and diabetes-prone KDP rats of both sexes were derived by mating these heterozygous rats (113). The rats were maintained under specific pathogen-free conditions. At 23 d, rats were weaned and given free access to either a diabetes-promoting cereal (CER)-based laboratory chow 5001 diet or a diabetes-retardant semi-purified AIN-93G hydrolyzed casein (HC) diet and monitored for T1D development until at least 200 days. Rats were studied within 48 hours of diabetes diagnosis or following 200 days without diabetes development.

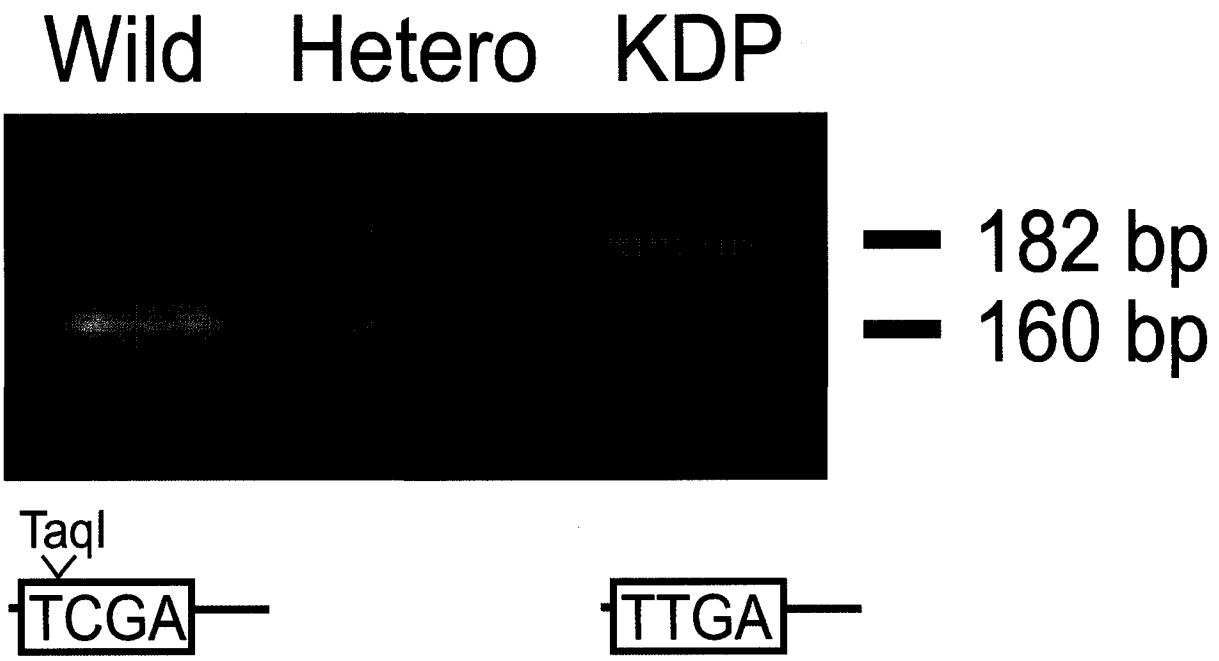
CHAPTER 4 – RESULTS

Genotyping the Offspring of Segregating Inbred KDP Rats

PCR-restriction fragment length polymorphism was used to genotype the offspring of segregating inbred KDP rats as described previously (Fig. 4-1; 113, 114). Wild-type rats were identified as homozygous for Cbl-b PCR product with an intact TaqI cleavage site, yielding a restriction cleavage fragment of 160 bp. Diabetes-prone KDP rats have a nonsense mutation in codon 455 of Cbl-b which disrupts a TaqI restriction site (114). These rats were identified as homozygous for Cbl-b PCR product with a mutated TaqI cleavage site, yielding a fragment of 182 bp. Mating between heterozygous rats yielded offspring in a ratio of approximately one wild-type to two heterozygous to one KDP rat, confirming that the presence of this mutant gene does not confer embryonic lethality (data not shown; 113).

Figure 4-1. Genotyping segregating inbred KDP rats by PCR-RFLP analysis of the Cbl-b gene

The Cbl-b gene was amplified by PCR with genomic DNA isolated from the offspring of segregating inbred KDP rats. The PCR products were digested with TaqI restriction enzyme and visualized by electrophoresis on 4 % agarose gel. Wild type (Wild) rats were identified as yielding a restriction fragment of 160 bp. Diabetes-prone KDP (KDP) rats, which have a mutated TaqI cleavage site, were identified as yielding a fragment of 182 bp. Heterozygous (Hetero) rats had both fragments.


Wild
Hetero
KDP
182 bp
160 bp
TaqI
TCGA
TTGA

Type 1 Diabetes Frequency

Wild-type and diabetes-prone KDP rats were weaned to either an HC or a CER diet and monitored for glucosuria. Fasting blood sugar was measured to confirm diabetes in rats with elevated glucosuria. None of the HC- or CER-fed wild-type rats developed T1D (Fig. 4-2). Consistent with previous reports, 90 % of CER-fed KDP rats developed T1D by 200 days. Over the same period, 80 % of HC-fed KDP rats developed T1D; a frequency that was not significantly different compared with CER-fed rats. The average age of diabetes onset was not significantly different between the two diet groups (Table 4-1).

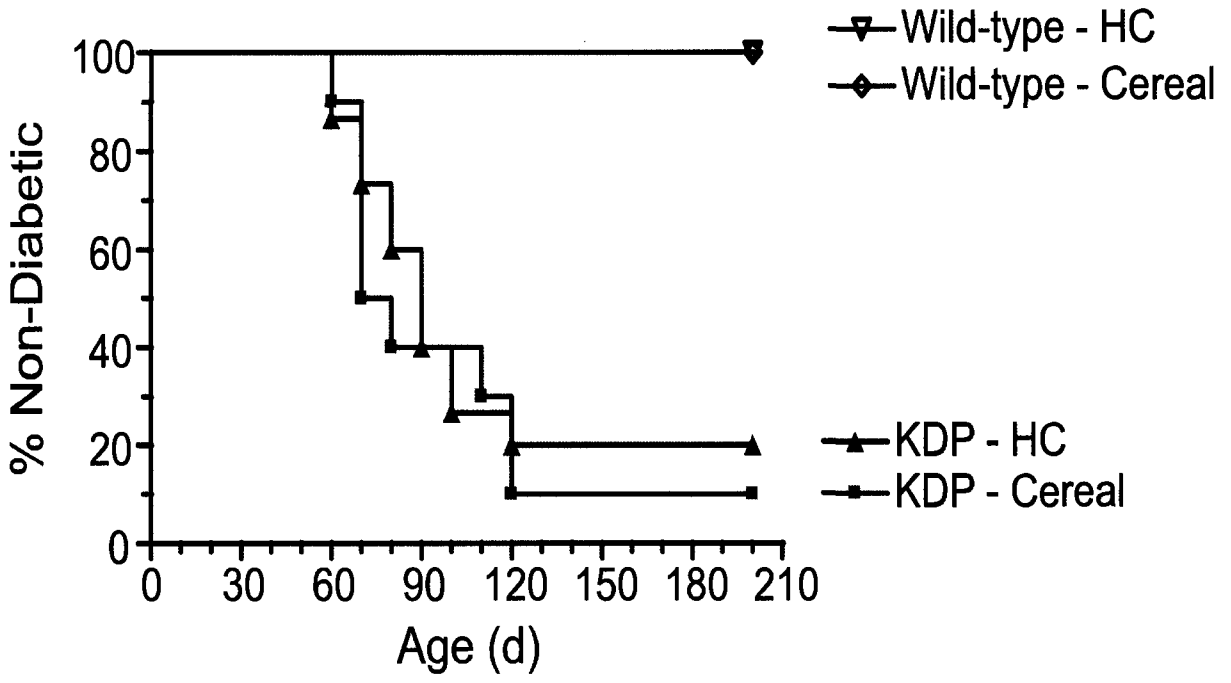
KDP and wild-type rats were weighed at diabetes development or otherwise at 200 days. There was no significant difference in body weight between HC-fed and CER-fed diabetic rats (Table 4-1). Because of the high incidence of T1D, only one asymptomatic CER-fed KDP rat was available for analysis.

Figure 4-2. Type 1 diabetes frequency is not modified by a hydrolyzed casein diet in KDP rats

Rats were weaned onto either an HC diet (n = 4 wild-type, 15 KDP) or a cereal-based diet (n = 4 wild-type, 10 KDP) at 23 d and monitored for the development of type 1 diabetes until 200 d. A Kaplan-Meier plot of % non-diabetic rats was generated. There was no significant dietary effect as determined by the log-rank statistical test.

Table 4-1. Age of diabetes onset and body weight in KDP rats

At diabetes onset (Diabetic) or approximately 200 days (Asymptomatic), animal age was recorded and HC-fed and cereal-fed, KDP and wild-type rats were weighed. Data represent the mean±SD.



MODEL	KDP				WILD-TYPE	
Diet	HC	CER	HC	CER	HC	CER
Diabetes	Diabetic		Asymptomatic		None	
Number (*n*)	8	3	3	1	4	4
Mean Age (d)	82 ± 18	81 ± 24	201 ± 6	200	208 ± 1	201 ± 1
Body Weight (g)	199.2 ± 53.7	239.7 ± 43.3	309.4 ± 125.3	214.8	355.3 ± 141.7	304.9 ± 98.9

Insulitis and Islet Number

Autoimmune destruction of pancreatic islet beta-cells begins with the accumulation of mononuclear cells at the islet periphery, termed peri-insulitis. This is followed by the infiltration of these cells into the islets and beta-cell destruction, termed insulitis.

The characteristics of KDP rat insulitis were determined (Fig. 4-3). Islets with peri-insulitis, insulitic infiltration and/or end-stage atrophic damage were quantified as a measure of islet damage. More than 90 % of diabetic KDP rat islets had insulitis, independent of diet (Table 4-2), and insulin^{+} cells were rarely observed in end-stage islets (data not shown). Asymptomatic diabetes-prone KDP rats had a low percentage of insulitic islets, an outcome which was not modified by diet and was similar to control rats (Table 4-2). Islet number was low in diabetic KDP rats, intermediate in asymptomatic KDP rats compared with wild-type rats, and was not significantly different between diets.

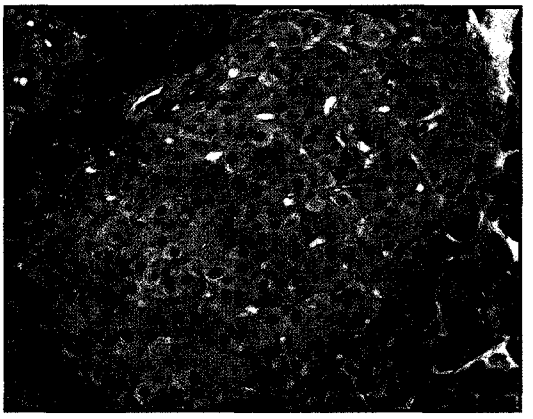
Figure 4-3. Characteristics of insulitis in the KDP rat pancreas

Hematoxylin and eosin-stained pancreatic sections showing (A) a healthy islet from an asymptomatic wild-type rat, (B) peri-insulitis and early infiltration of darkly stained mononuclear cells into the islet of a diabetic KDP rat, (C) mononuclear cell infiltration (insulitis) in the islet of a diabetic KDP rat, and (D) an end-stage infiltrated and damaged islet of a diabetic KDP rat. Bar = 50 μm.

Table 4-2. Islet damage score in KDP rats

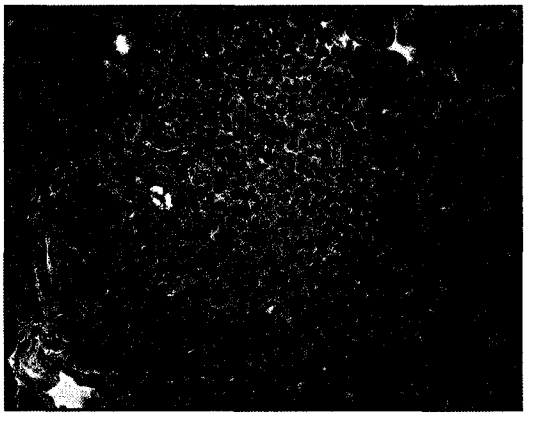
Haematoxylin and eosin-stained pancreas sections were prepared following diabetes onset (Diabetic) or approximately 200 days (Asymptomatic) from HC-fed and cereal-fed, KDP and wild-type rats, and analyzed by light microscopy. Insulitis was scored as the percentage of insulitic and/or damaged islets on one section per animal. Islet number, normalized to section area, was determined on one section per animal. The number of animals studied is indicated and data represent the mean±SD.

(A) Healthy Islet

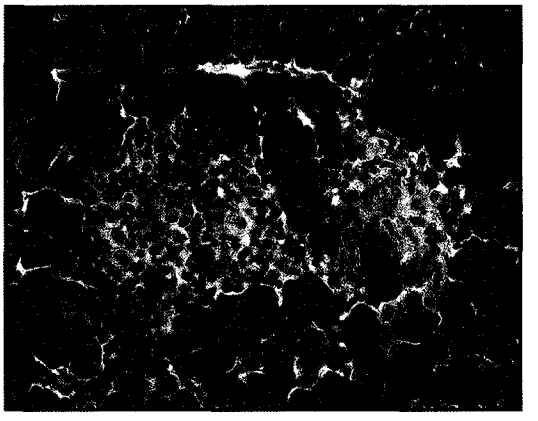


(B) Peri-insulitis

(C) Insulitis



(D) Damaged Islet



MODEL	KDP				WILD-TYPE	
Diet	HC	CER	HC	CER	HC	CER
Diabetes	Diabetic		Asymptomatic		None	
Number (*n*)	8	4	3	1	4	4
Insulitic Islets (%)	94.6 ± 6.4	95.2 ± 5.5	23.5 ± 20.6	31.7	14.0 ± 10.5	13.2 ± 10.5
Islet Number (Islets/cm^2)	21.4 ± 8.3	31.6 ± 12.0	47.6 ± 22.4	50.0	71.3 ± 21.7	54.6 ± 25.5

Pancreatic Tubular Complexes

Tubular complexes are rare duct-like regions in the pancreas which typically arise in response to pancreatic damage (121). They consist of several closely associated duct-like structures, consisting of low cuboidal or flattened cells surrounding a large lumen, embedded in connective tissue (121). These structures have been proposed to derive from the de-differentiation of acinar tissue and may be a source of islet neogenesis (167, 168). Pancreatic tubular complexes were identified in both KDP and wild-type rats and some contained insulin$^+$ cells (Fig. 4-4). There was no significant dietary effect on frequency of these structures (Table 4-3).

Figure 4-4. Pancreatic tubular complexes in KDP rats

Haematoxylin and eosin-stained pancreas sections were prepared and analyzed by light microscopy. Serial sections were used for immunohistochemical insulin staining and were counterstained with hematoxylin. (A, B) A lobular tubular complex from a 200 d asymptomatic HC-fed KDP rat. (C, D) A tubular complex containing insulin^{+} cells from a 200 d asymptomatic cereal-fed wild-type rat. Bar = 500 μm in A; 100 μm in C; 50 μm in B, and D.

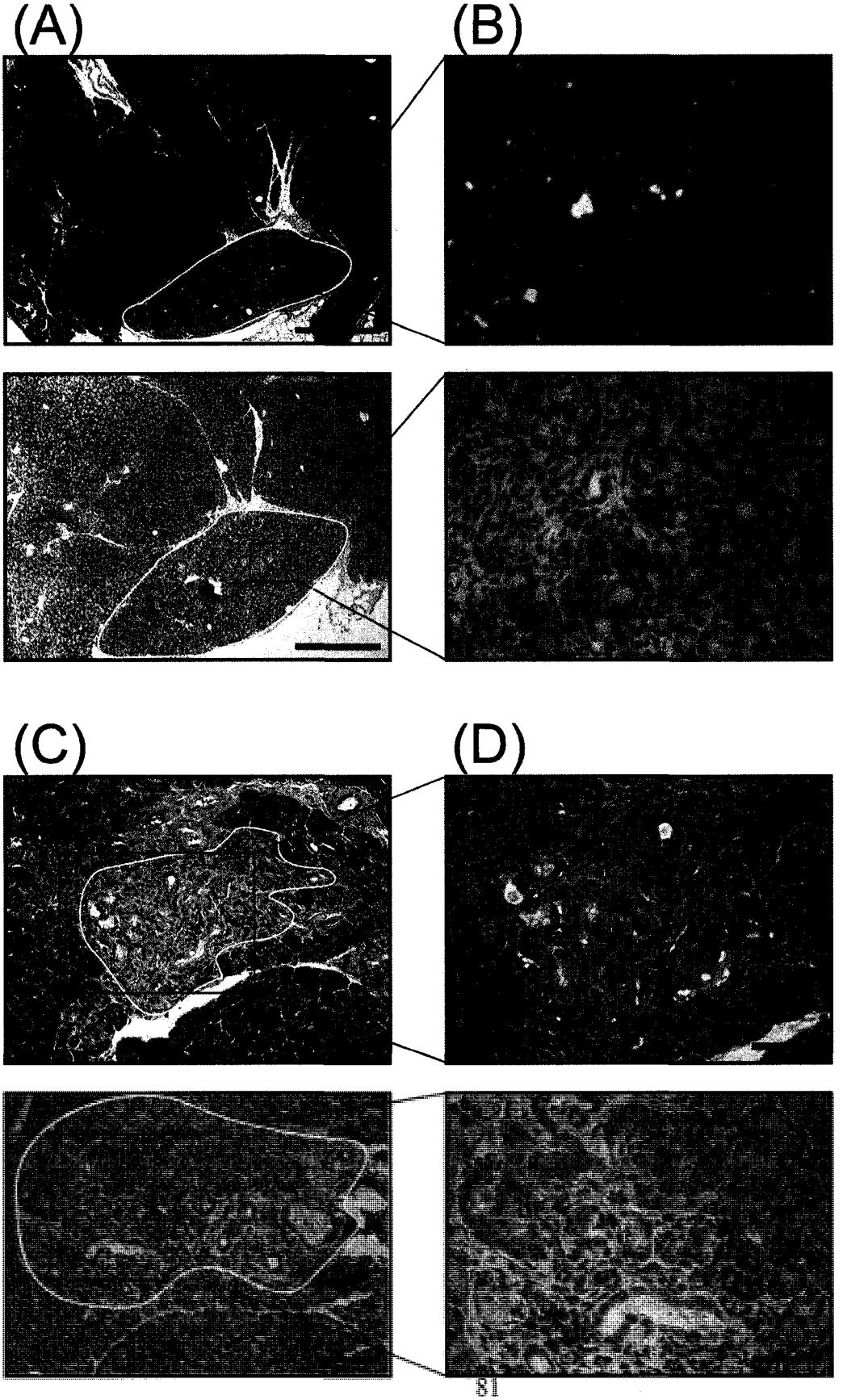

(A)
(B)
(C)
(D)

Table 4-3. Tubular complex score in KDP rats

Haematoxylin and eosin pancreas sections were prepared at diabetes onset (Diabetic) or 200 days (Asymptomatic) from HC-fed and cereal-fed, KDP and wild-type rats, and analyzed by light microscopy. The number of tubular complexes on one section per animal was quantified.

MODEL	KDP				WILD-TYPE	
Diet	HC	CER	HC	CER	HC	CER
Diabetes	Diabetic		Asymptomatic		None	
Number of Rats with Tubular Complexes On A Section of Pancreas	1/8	0/4	2/3	0/1	0/4	1/4

CHAPTER 4 – DISCUSSION

A cereal-free hydrolyzed casein-based diet that retards T1D in BBdp rats and NOD mice did not protect diabetes-prone KDP rats from type 1 diabetes or islet damage, demonstrating that autoimmune diabetes resulting from dysfunction of the T cell regulator Cbl-b is not modifiable by diet (Fig. 4-2). Rats on the CER-based diet developed a cumulative T1D frequency of 90 % by 200 d (Fig. 4-2) with an average age at onset of 81 days (Table 4-1), similar to previous reports (113, 114, 165, 166). None of the wild-type rats, which have functional Cbl-b, developed T1D. Similar to the KDP rat, two other unique rat models of T1D were not protected from diabetes by hydrolyzed casein-based or amino acid diets. The first is the diabetes-resistant BB rat introgressed with the lymphopenia gene (*Ian5/GiMap5*) from diabetes-prone rats (169). The second is the diabetes-resistant BB rat treated with anti-ART2 antibody and poly I:C to remove regulatory T cells (170). These animals, like the KDP rat, develop diabetes as a result of unique immune defects or alterations and probably represent mainly immune-mediated forms of diabetes which are not strongly influenced by dietary modification.

T cell activation signals are transduced by the T cell receptor (TCR)-CD3 complex in response to recognition of cognate antigen, and by the CD28 co-stimulatory receptor upon recognition of co-stimulatory molecules (171). The resultant cell signaling pathways, such as phosphatidylinositol 3-kinase (PI3-k), converge at the phosphorylation and activation of the GTP/GDP exchange factor Vav, which modulates T cell activation (172, 173). Cbl-b is an E3 ubiquitin ligase that negatively regulates and sets the threshold for T cell activation by ubiquitinating phosphorylated Vav and PI3-k, targeting them for proteasomal degradation (174-178). The CD28 co-stimulatory signal allows T cells to overcome this threshold by

inducing the ubiquitination and degradation of Cbl-b, allowing the accumulation of activated Vav protein (179, 180). In Cbl-b knock-out rodents, Vav remains highly activated, and co-stimulatory signaling through CD28 is no longer required for T cell activation, resulting in a hyperproliferative T cell phenotype upon recognition of antigen alone (174-176). In the KDP rat, a nonsense mutation in Cbl-b results in an inactive truncated form of this protein. Homozygosity for this mutated gene results in T cell activation in the absence of co-stimulatory signaling and is associated with the development of autoimmune lymphocyte infiltration into multiple tissues including thyroid gland, submandibular gland, kidney, adrenal and pituitary glands, and pancreatic islets (114). The islet infiltration results in the development of T1D (165). Similar to the KDP rat, when the Cbl-b was knocked out in the RIP-gp transgenic model, diabetes autoimmunity was enhanced (181). The lack of dietary modification of KDP diabetes suggests the effects of the Cbl-b mutation act downstream of the protective effect of the HC diet in BBdp rats and NOD mice.

Silent polymorphic variations of the Cbl-b gene have been identified in patients with T1D (182-184). While two studies found no association, another found that one of these silent polymorphisms was associated with T1D risk (182). Thus the involvement of Cbl-b as a human T1D risk gene remains unclear.

Insulitis has previously been characterized in KDP rats and in the diabetes-prone LETL strain from which the KDP rat was derived. The present results confirm that most of the islets in diabetic KDP rats are heavily infiltrated with mononuclear cells or are atrophic with little mononuclear cell infiltration following beta-cell destruction (Table 4-2; 114, 165, 166). This insulitis is associated with a decrease in islet number (Table 4-2) and size, and with a reduction in beta-cell number (185). The present data are consistent with previous

findings (114, 165, 166) and expand the data by demonstrating that intermediate insulitis in asymptomatic KDP rats is associated with reduced islet numbers compared with controls, and that insulitis is not modified by diet in KDP rats (Table 4-2; Lefebvre *et al, in preparation*).

Pancreatic duct-like tubular complex regions containing insulin$^+$ cells (which have been proposed to be a source of islet neogenesis; 121) were identified in both KDP rats and the corresponding wild-type strain (Fig. 4-4). Thus, while the KDP rat did not prove to be a good model of dietary modification of T1D (186), it could be a good model of the regenerative processes which can occur in the pancreas (121). Unlike the KDP rat, diabetes in the BBdp rat model resembles human diabetes in that it requires multiple risk genes for development. BBdp diabetes is strongly influenced by diet, however the role of microbes in this process is unknown.

CHAPTER 5 – Parsing the Role of Microbes and Diet in the Development of Autoimmune Diabetes in Diabetes-prone BioBreeding Rats

CHAPTER 5 – INTRODUCTION

Cereal (CER) protein-based diets promote the development of type 1 diabetes in BBdp rats (57) while an HC-based diet significantly protects from diabetes (57). While diet modifies both gut microflora and T1D incidence, it is unclear whether microbes are involved in the natural course of diabetes pathogenesis and in dietary modification of this process.

The gastrointestinal tract is the primary interface between dietary antigens and the immune system. Diet does not modify BBdp rat histological gut damage, but does modify inflammatory Th1 cell activation and possibly gut permeability (Fig. 2-2; 62, 85, 88). Gut damage and permeability pre-date and correlate with diabetes development and could play a role in disease pathogenesis (87-89). Markers of gut damage and inflammation in BBdp rats include increased numbers of intraepithelial lymphocytes, as well as CD4 T helper cell infiltration in the lamina propria (89), and increased IFN-γ expression in the gut and draining mesenteric lymph nodes (Figures 2-1 and 2-2).

Studies in germ-free (GF) animals which have no commensal or pathogenic microbes revealed that commensal gut microflora have a major influence on intestinal structure and function and are critical for the development of the mucosal immune system. GF rats have immature Peyer's patches, low MHC class II expression on intestinal epithelial cells, reduced numbers of intraepithelial lymphocytes, lamina propria regulatory T cells, and B cells, and reduced mucosal IgA and serum IgG antibody levels (117, 118, 187-189). The gut of these rats also display cecum enlargement, reduced motility, and an increased concentration of

brush border enzymes. The gastrointestinal tract of standard specific pathogen-free (SPF) laboratory rodents is maintained free from a defined relatively small collection of bacteria, viruses and other pathogens, but is colonized by a vast array of commensal micro-organisms comprised of bacteria, some viruses, fungi, and protozoa, which are usually not harmful (118). Under some conditions, commensal microflora can induce chronic inflammatory and autoimmune diseases (118).

Levels of selected bacteria were higher in the gut of diabetic compared with asymptomatic BBdp rats and NOD mice, raising the possibility that the status of the gut microflora is related to T1D risk (190-192). Oral administration of fuscidic acid, which has both anti-staphylococcal and immunosuppressive properties, or of Bactrimel and colistine sulphate antibiotics, reduced diabetes incidence in BBdp rats (192, 193). Diabetes can be promoted by components and products of bacteria which can be encountered in the diet, such as lipopolysaccharide from gram-negative bacteria, enterotoxins from *staphylococci*, and streptozotocin and bafilomycin A1 from *Streptomyces* species (38, 194-197). Diabetes is also promoted by zonula occludens toxin from *Vibrio cholerae* which mimics mammalian zonulin and induces gut permeability (87).

Microflora have a role in the metabolism of gut lumen contents (118), and can thus modify nutrients in the gut (198, 199). Conversely, diet can modify certain populations of gut microflora (117). For example, a diabetes-promoting cereal-based diet increased the number of gram-positive caecal bacteria in NOD mice (190). Protection from diabetes in BB rats treated with antibiotics, which have reduced gut bacteria, was enhanced when combined with a hydrolyzed casein diet (192). However it is unclear if this was due to a secondary effect of the antibiotic treatment. Other reports have demonstrated no difference in diabetes

incidence in BB rats under gnotobiotic conditions, where the composition of the microflora is defined (200), and following wet hysterectomy (201). However, these studies were not performed under rigorously monitored, strict GF conditions and rats were fed strongly diabetes-promoting cereal-based diets. This leaves the question of whether dietary modification of T1D is attributable to interaction with gut bacteria. We hypothesized that dietary modification of T1D is altered by the presence of microbes.

CHAPTER 5 – EXPERIMENTAL DESIGN

To separate the effects of microbes and diet, male and female BBdp rats were derived and maintained under standard specific pathogen-free (SPF) or strict germ-free (GF) conditions. Pups were weaned at 23 d and given free access to either an AIN93G hydrolyzed casein (HC) diet or a cereal (CER)-based NTP-2000 diet. Rats were monitored for T1D development until at least 134 days. SPF results were pooled from two studies performed before and after the GF study. Gut immune inflammatory status was analyzed by RT-PCR in MLN, from 134-149 d non-diabetic rats.

CHAPTER 5 – RESULTS

Type 1 Diabetes is Modified by Diet and Microbes in Germ-free BBdp Rats

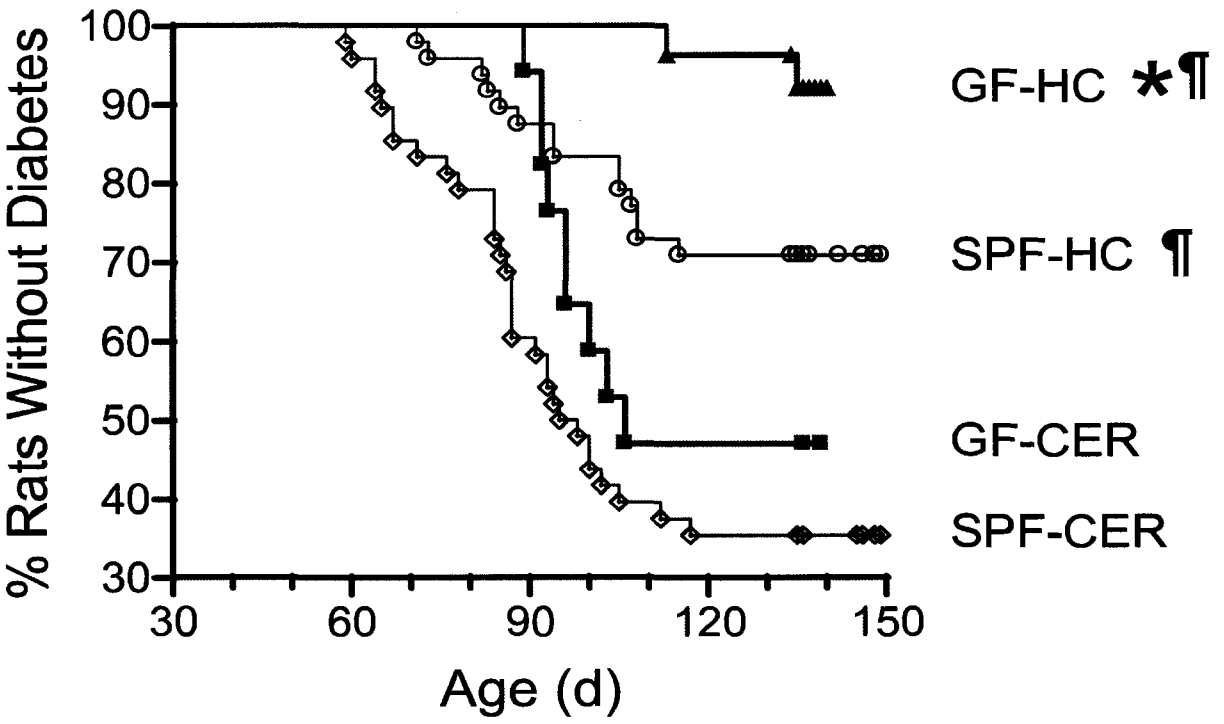
An HC diet protected rats from diabetes compared with a CER diet regardless of the presence or absence of microbes ($p<0.0005$; Fig 5-1). GF-CER rats developed diabetes at a final incidence of 53 % by 134 days. This was lower, but not significantly different from the 65 % incidence observed in SPF-CER rats. The diabetes-modifying effect of microbes was most apparent in HC-fed rats. GF-HC rats were almost completely protected from diabetes with a 7 % incidence, which was significantly less than the 29 % incidence in SPF-HC rats ($p<0.05$). Diabetes onset (Table 5-1) was significantly delayed in GF compared with SPF animals. It was also significantly delayed in GF-HC compared with GF-CER rats. Body mass and pancreas mass were not modified by microbes or diet (data not shown). Subsequent analyses were performed on rats which remained asymptomatic until 134-149 days to avoid the confounding effect of end-stage autoimmune diabetes.

Figure 5-1. Effect of diet and microbes on type 1 diabetes development in BBdp rats

BBdp rats were maintained in SPF or GF conditions, fed HC- or CER-based diets, and monitored for T1D development. A Kaplan-Meier plot of % non-diabetic rats was generated. GF-HC (n=27), SPF-HC (n=48), GF-CER (n=17), SPF-CER (n=48). * indicates a statistically significant difference (p<0.05) between GF and SPF rats; ¶ indicates a statistically significant difference (p<0.0005) between HC-fed and CER-fed rats; as determined by the log-rank test.

Table 5-1. Age of diabetes onset in BBdp rats

Mean age of diabetes onset for HC- and CER-fed BBdp rats under GF and SPF conditions. Values represent mean±SD. Statistical differences were determined by ANOVA followed by LSD pos-hoc analysis.



DIET	HC		CER	
BBdp rat	GF	SPF	GF	SPF
Total number of rats per group (*n*)	27	48	17	48
Number of diabetic rats per group (*n*)	2	14	9	31
Age of diabetes onset (d)	124 ± 15	94 ± 14	96 ± 5	85 ± 15
ANOVA	$p<0.05$, vs. HC-SPF	-	$p<0.05$, vs. CER-SPF $p<0.05$, vs. HC-GF	-

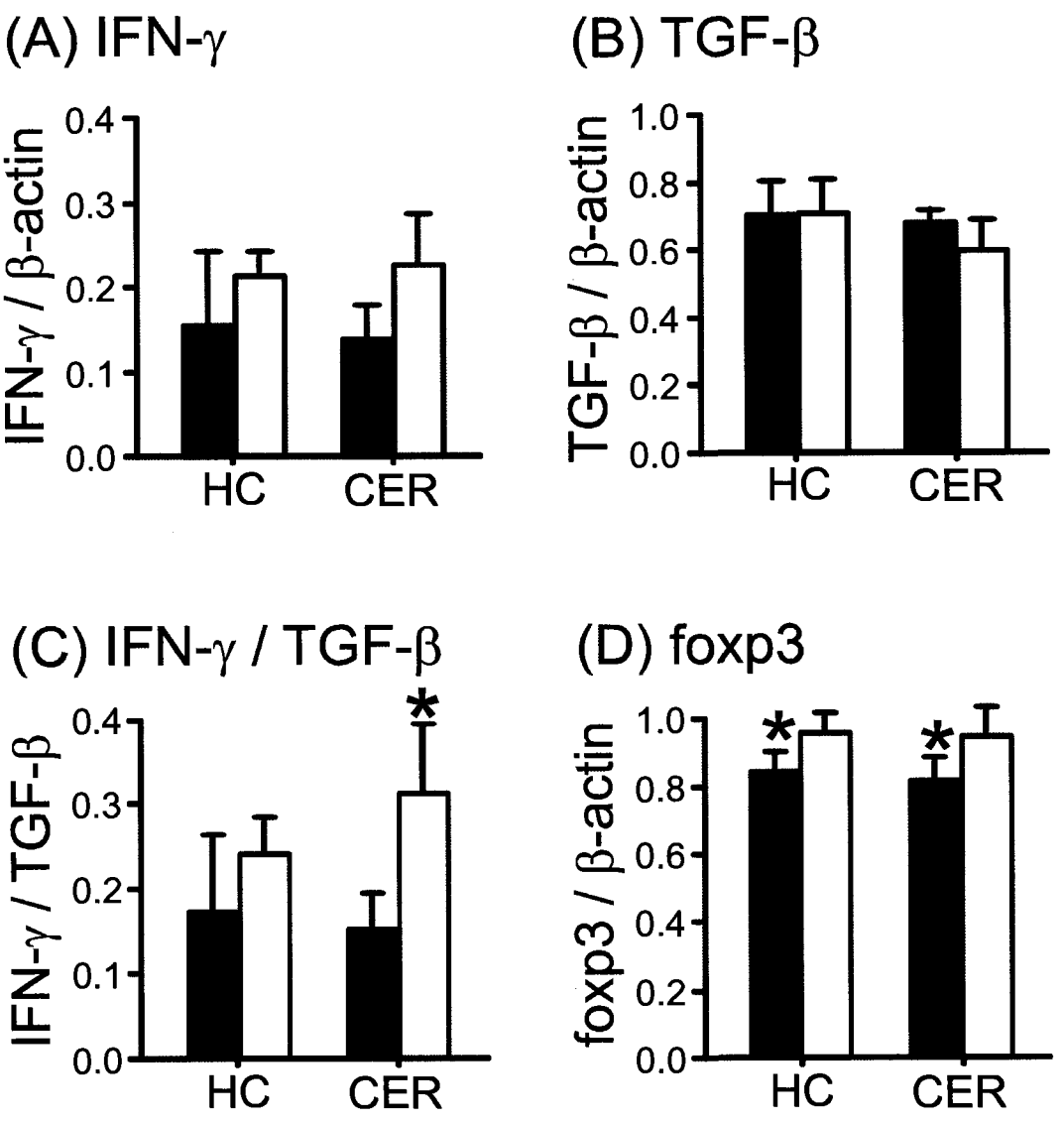
Immune Cell Marker Expression in Gut-associated Lymphoid Tissues

There was a trend of increased gene expression of the Th1 cytokine IFN-γ in the MLN of SPF compared with GF rats (Fig. 5-2A). This was significant in diabetic rats (data not shown). Expression of the tolerizing Th3 cytokine TGF-β was not modified (Fig. 5-2B). This expression pattern resulted in a significantly higher IFN-γ/TGF-β ratio in SPF-CER compared with GF-CER rats and a similar trend in HC-fed rats (Fig. 5-2C). IFN-γ/TGF-β ratio was highest in the MLN of rats with the most diabetes-promoting combination of SPF-CER conditions (Fig 5-2C). The ratio of IFN-γ to the Th2 cytokine IL-5 displayed similar trends (data not shown).

Surprisingly, despite the lack of bacterial stimulation in GF rats, there were no differences in the gene expression of the bacterial lipopolysaccharide-receptor, TLR-4 or of its downstream signaling molecule MyD88 (data not shown). Expression of the regulatory T cell-specific marker foxp3 was not modified by diet but was slightly lower in MLN from GF compared with SPF rats (Fig. 5-2D).

Figure 5-2. Gene expression in mesenteric lymph nodes of BBdp rats

Gene expression of IFN-γ (A), TGF-β (B), IFN-γ/TGF-β ratio (C), and foxp3 (D) measured by semi-quantitative RT-PCR in freshly isolated MLN tissue of HC and CER-fed asymptomatic BBdp rats under GF (closed bars) and SPF (open bars) conditions. Data were normalized to β-actin and represent mean±SD. GF-HC (n=8), SPF-HC (n=3), GF-CER (n=5), SPF-CER (n=3). * indicates a statistically significant difference (p<0.05) between GF and SPF rats as determined by ANOVA followed by LSD post-hoc analysis.


(A) IFN-γ
IFN-γ / β-actin
0.4
0.3
0.2
0.1
0.0
HC
CER
(B) TGF-β
TGF-β / β-actin
1.0
0.8
0.6
0.4
0.2
0.0
HC
CER
(C) IFN-γ / TGF-β
IFN-γ / TGF-β
0.4
0.3
0.2
0.1
0.0
*
HC
CER
(D) foxp3
foxp3 / β-actin
1.0
0.8
0.6
0.4
0.2
0.0
*
*
HC
CER

CHAPTER 5 – DISCUSSION

The two most studied environmental influences on development of T1D are infectious agents and diet. However, it is unclear which is the main influence and whether they interact to affect development of T1D. To address these questions, GF and SPF BBdp rats were compared to distinguish dietary from microbial influences. A CER-based diet promoted T1D whereas an HC-based diet almost completely protected GF BBdp rats (Fig. 5-1). This is the first demonstration that a CER-based diet both accelerates and promotes diabetes development compared with an HC-based diet in BBdp rats under strict GF conditions. In GF rats, diabetes was delayed and incidence was lower than rats maintained under SPF conditions, similar to the effect of antibiotic treatment (192). This reduced incidence in GF rats was only significant in HC-fed rats, where CER antigens are absent. Thus, both diet and microbes are environmental factors determining diabetes outcome in the Ottawa BBdp rat colony, with the major effect coming from diet. These results are in contrast to results from NOD mice, where GF and gnotobiotic conditions were associated with an increased frequency of diabetes (202).

GF conditions lead to a poorly developed gut immune system which lacks T cells (189, 203). This could be part of the mechanism for reduced diabetes incidence in GF rats. Indeed, analyses performed by Dr. Gen-Sheng Wang and Mr. Christopher Patrick in the Scott lab, identified higher $CD3^+$ intraepithelial lymphocyte (IEL) numbers in the gut mucosa of SPF compared with GF BBdp rats. Similar to celiac patients (204), the IELs were also increased in rats fed a diet containing CER proteins. The cecum of GF BBdp rats was enlarged compared with SPF rats (data not shown), consistent with previous reports of increased luminal contents in GF rats due to decreased fermentation of mucus (118, 205).

This, in combination with the reduced IEL T cells and reduced regulatory T cell-specific foxp3 expression (Fig. 5-2D), is characteristic of GF rats, providing further support that the rats lacked microflora (189, 206, 207). Routine microbiological testing confirmed that the rats were germ-free.

Inflammatory Th1 cell activation has been shown to be induced by a CER-based diet in the gut of SPF BBdp rats (Fig. 2-2) and NOD mice (95). Bacteria can also promote the expression of Th1 cytokines in the gut (117, 208). Both diet and microbes had a similar Th1 cytokine-promoting trend in the present study (Fig. 5-2). While the Th1-promoting effect of a CER diet was not significant in the MLN of the older (>134 d) asymptomatic BBdp rats, as it was in the gut of 45 day BBdp rats (Fig. 2-2), there was a trend towards an increased IFN-γ/TGF-β ratio in CER-fed rats (Fig. 5-2C). Future studies in young GF BBdp rats will be useful to clarify the interaction between microbes and diet in the period before diabetes.

A Th1-promoting effect of bacteria was observed. SPF rats had increased IFN-γ expression in the MLN compared with GF rats (Fig. 5-2A). The decreased Th1 cytokine expression in GF rats could result from the immature mucosal immune system or from a lack of the diabetes-promoting effects of bacteria. The reduced gut T cell numbers and decreased inflammatory Th1 status in the MLN under GF conditions and an HC diet parallel reduced islet autoimmunity and a delayed and reduced diabetes incidence.

Expression of the regulatory T cell-specific transcription factor, foxp3, was slightly lower in GF BBdp rats (Fig. 5-2D), consistent with the requirement of intestinal bacteria for the development of regulatory T cells (207, 209). However, the minor differences in foxp3 expression were likely not biologically relevant.

TLR-4 and the downstream signaling molecule MyD88 are upregulated in immune cells in response to bacterial lipopolysaccharide (210) and in response to the inflammation associated with colitis (211). TLR-4-expressing antigen presenting cells in the MLN can be resident in this tissue or can migrate from the gut (79, 212). TLR-4 expression has not been studied in any model under GF conditions. Surprisingly, expression of TLR-4 and MyD88 was not modified under GF conditions. Thus, the genes for bacteria-sensing molecules were unchanged despite the lack of signals from bacteria.

A cereal-based diet promoted T1D, and in its absence a diabetes-promoting effect of microbes was revealed. The reduced gut IEL number and reduced MLN Th1 cytokine levels in GF rats was consistent with the delayed T1D development and reduced T1D incidence. Thus, diet is a major factor influencing the development of T1D under both SPF and GF conditions, and microbial factors modify the development of autoimmune diabetes in the absence of diabetes-promoting cereal antigens.

GENERAL DISCUSSION

T1D is a complex disease. Interactions with environmental factors probably contribute to its expression. Animal models of diabetes provide a useful testing ground for identifying and characterizing the effects of candidate environmental factors. Gut damage is a constitutive feature in diabetes-prone BB rats (89). Consistent with this histological damage, specific inflammatory markers are increased. These include peroxidase (85, 89) and the ratio of T-bet/GATA-3 (Fig. 2-1). The transcription of Th1/Th2 cytokine expression is controlled mainly by T-bet and GATA-3 (Chapter 1). In confirmation of the effect of these transcription factors, T cells expressing IFN-γ were increased in the MLN of diabetes-prone BB rats (109) which also displayed an increased T-bet/GATA-3 ratio (Fig. 2-1). IFN-γ, the principal effector cytokine of the Th1 cell-mediated immune response, normally promotes the eradication of intracellular pathogens including bacteria, parasites, yeasts, and viruses. This response is often associated with inflammation. IFN-γ recruits inflammatory leukocytes, induces cytokine secretion by macrophages, and enhances the microbicidal activity of phagocytes, cytotoxic T cells, and NK cells (6).

I tested if the inflammatory immune status of the gut was related to dietary protein source. A cereal-based diet induced Th1 cytokine production in the gut and mesenteric lymph nodes of diabetes-prone BB rats (Fig. 2-2). Similar findings were also found in NOD mice (213). Cells expressing Th1 cytokines were reduced in diabetes-prone rats fed a low-antigen, hydrolyzed casein diet, suggesting that these cells are activated by a component of the cereal-based diet (Fig. 2-2; 109).

The gastrointestinal tract is drained by the MLN, the main site of T cell maturation in the gut immune system (79). Overall cell proliferation as well as metabolic activity were previously found to be increased in the BBdp rat MLN (92). Consistent with this finding, the increased level of Th1 cytokines in the MLN of cereal-fed BB rats (Fig. 2-2) reflected the unusually high response of CD4 T cells to wheat (Fig. 2-4). The activation and maturation of these T cells occurred in an environment rich in antigen-presenting cells (109) but low in immunosuppressive T regulatory cells (Fig. 2-5; 35, 44).

The histological appearance of gut damage itself was not improved in rats fed a diabetes-retardant HC diet in BB rats (89) suggesting this is a genetically determined trait. While histological damage itself is not modified by diet, it could still be an initial required permissive co-factor which allows the functional activation of other diet-related inflammatory processes in the gut (87, 88).

Increased gut permeability predates diabetes in BB rats (84, 85). Increased permeability is also present in T1D patients (80-83). Altered permeability in diabetes-prone individuals could allow abnormal exposure to dietary antigens. This, in combination with the inflammatory immune status of the gut, could disrupt oral tolerance to certain dietary proteins (62).

Gut permeability was increased in BBdp rats fed a diabetes-promoting, cereal-based diet (85, 88), as was IFN-γ expression (Fig. 2-2). This infers that IFN-γ expression could be promoting the breach in the gut barrier similar to the pathogenesis of celiac disease and other chronic gut inflammatory conditions that are associated with Th1 cytokine responses and damage to the gut (108, 146). The altered permeability could involve IFN-γ-induced downregulation of tight junction proteins in intestinal epithelial cells (214). By this

mechanism, the Th1 bias in the BBdp rat gut could further increase gut permeability, allowing greater exposure to wheat antigens, inducing more Th1 cytokines, and creating a positive feedback mechanism of gut inflammation.

Increased gut permeability has been directly linked with the development of diabetes in BBdp rats by an unknown mechanism (87). The HC diet has been suggested to protect BB rats and NOD mice from diabetes either due to the presence of diabetes-retardant components or to the lack of diabetes-promoting cereal proteins (2). These cereal proteins could be involved in diabetes pathogenesis (57). This hypothesis suggests that the high incidence of T1D in BBdp rats exposed to dietary wheat could involve an immune reaction to wheat proteins. The development of enteropathy in the gut of pre-diabetic BB rats (89) followed shortly thereafter by increased permeability (84, 85), could allow increased exposure to soluble dietary wheat (and other) antigens and set the stage for continuing inflammatory Th1 damage. Wheat-reactive T cells activated in the gut have the same inflammatory Th1 phenotype as autoreactive T cells in the pancreas (39-41). Thus the inflammatory immune state of the gut could modify the immune attack on the pancreas and could be one mechanism by which gut damage promotes T1D (215).

It remains to be determined whether or not the dietary effect on diabetes incidence involves the action of beta-cell reactive immune cells in the gut. It is alternatively possible that immune reactivity to cereal proteins may not be related to diabetes. It could simply be a reflection of dysregulation in the gut immune system, or to a lack of oral immune tolerance in diabetes-prone individuals (140). It could also be a secondary effect of T1D pathogenesis. It is, however, possible that antigenic wheat proteins induce immune cells that traffic to the pancreas and promote beta-cell destruction. Adoptive transfer experiments in BB rats and in

NOD mice have shown that cells isolated from the MLN traffic to the pancreas where they can promote T1D (98, 149, 216). The mechanism could involve cross-reactivity with a self antigen or bystander activation of beta-cell-specific immune cells.

Molecular mimicry is thought to occur when a lymphocyte is unable to distinguish between structural similarities in self and non-self antigens (217). If activated by non-self antigens, such a lymphocyte can cause autoimmunity. There are, however, few definitive examples of molecular mimicry (218, 219). Lymphocytes activated by a dietary antigen could trigger or accelerate the autoimmune attack on beta-cells if there is molecular mimicry between a dietary antigen and a beta-cell-specific self antigen (57, 218). For example, it has been reported that there may be molecular mimicry between IA-2 (a T1D autoantigen) and wheat- or soybean-derived NADH ubiquinone (220). It was also hypothesized that there was molecular mimicry between an epitope, termed ABBOS, in bovine serum albumin, found in cow milk and beef, and the beta-cell protein ICA69 (221). However, other reports disputed this and could not find cross-reactivity (222, 223). An immune response to cow insulin in milk, which has a similar sequence to human insulin, has also been suggested to promote diabetes (224).

Bystander lymphocyte activation is another mechanism by which an immune response to dietary protein could modify T1D autoimmunity. This mechanism has been proposed to occur when the immune response to a non-self antigen results in the activation of an unrelated autoimmune lymphocyte which happens to be present in the same micro-environment (159). Beta-cell-specific immune cells that circulate through the gut in parallel with cells undergoing immune reaction to dietary cereal could be activated in this way.

To date, the identity of individual diabetes-related proteins is still unclear, and mechanisms of action are unknown. There is some evidence that the diabetes-promoting activity in wheat could be associated with the low-molecular-weight glutenin fraction (56). Other studies identified a wheat Glb1 homologue as a diabetes-related antigenic wheat globulin protein in BB rats, NOD mice, and humans (57, 225). Antibody reactivity to Glb1 correlated with islet inflammation and damage in BBdp rats (57). Antibody and T cell reactivity to this protein was also detected in a patient with T1D and CD described in Chapter 3 (153).

There are indications that dietary protection from T1D acts not only by effects on T cells, but also in part by affecting islet regeneration. Rats fed a hydrolyzed casein diet have an increased beta-cell fraction; which could in part be related to islet neogenesis (226). Patients who followed a wheat-free diet for 6 months exhibited increased first phase insulin response to glucose which reflects beta-cell mass, supporting the theory that avoiding dietary wheat has a beneficial effect of protecting beta-cell mass (77). However unlike BBdp rats (Fig. 5-1) and NOD mice (61), a cereal-free hydrolyzed casein-based diet did not protect diabetes-prone KDP rats from diabetes. These animals represent a unique model of largely immune-driven T1D.

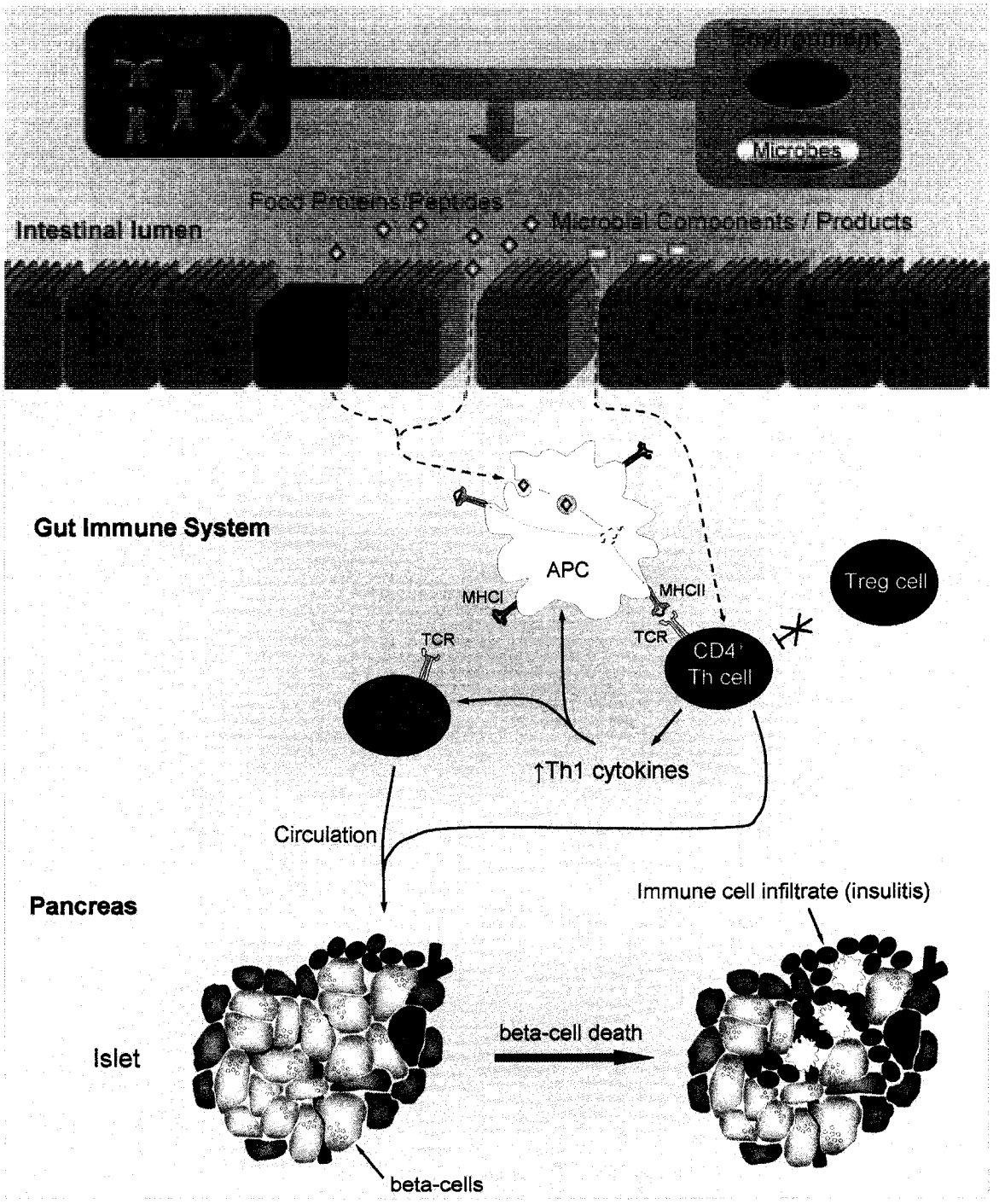
Thus results from two of three animal models of T1D reflect the possibility that dietary cereal proteins could affect the expression of diabetes in a subset of genetically susceptible humans. It is difficult to identify environmental factors which modify diabetes incidence in humans because of the difficulty of associating past exposure with disease development. There is, however, evidence that dietary proteins could be one of these factors. Recent findings support the proposal that cereals are possibly involved in the development of diabetes autoimmunity in high risk children and that infants fed a hydrolyzed casein-based formula are less prone to develop diabetes autoantibodies (75, 76).

Celiac disease results from Th1 cell-mediated autoimmune damage to the gut mucosa triggered by gliadin peptides in gluten from grains such as wheat (111). In addition to the shared HLA risk alleles, gut barrier dysfunction and exposure to wheat gluten could be common risk factors for both CD and T1D (2). This link is supported by a high coincidence of CD in patients with T1D (1-8%; Refs. 150, 156). The majority of patients diagnosed with both diseases develop T1D first. It is possible that the gluten-free diet implemented as the standard treatment for celiac disease could decrease the risk of developing T1D. The present work identified a specific cellular immune response to wheat proteins in patients with T1D without clinical signs of CD. It remains to be determined if the reactivity to wheat develops as a secondary complication of diabetes or if it is present in the pre-diabetes period.

T1D likely develops by different pathways in patients that fall into various sub-categories of T1D. According to this model, factors such as genetic susceptibility, the status of multiple organ systems, and exposure to several environmental variables at different ages and doses, affect processes which promote or inhibit diabetes pathogenesis in each individual. As described in the summary model (Figure 6-1), there is damage to the gut epithelium in a subset of humans and in animal models with genetic susceptibility for T1D. This could permit an unusually large concentration of luminal contents to enter the GALT. These luminal components, including dietary proteins/peptides and microbial agents, could then promote an immune reaction. Dietary protein-specific $CD4^+$ T cell activation was identified in BB rats and in patients with T1D. $CD8^+$ T cells were also activated in patients. It is speculated that the immune response to dietary antigens modifies the immune attack on beta-cells in the pancreas by a direct or bystander mechanism. Thus, diabetes-prone individuals whose gut barrier is damaged and whose immune system is predisposed to target beta-cells most likely do not have a normal response to dietary antigens.

Figure 6-1. Environmental modification of gut immune status in individuals at genetic risk of developing T1D

The gut intestinal epithelial cell (IEC) barrier is compromised in some T1D patients and in diabetes-prone rodents. This could be related to the opening of tight junctions. Increased permeability could permit abnormal exposure of the gut immune system to cereal or microbe constituents. Lumen peptides can also gain access the mucosa by uptake through microfold (M) cells. Antigen-presenting cells (APC) can take up these exogenous antigens and present them to CD4 T cells on MHC class II. Activated CD4 T cells secrete pro-inflammatory Th1 cytokines; inducing CD8 T cells and APCs. Immunosuppressive regulatory T cells are reduced and unable to maintain tolerance. Activated lymphocytes can enter the systemic circulation and can migrate to the pancreatic islets at ages which pre-date diabetes development. Thus, dietary proteins could play a role in gut inflammation and destruction of β-cells by a mechanism involving compromised gut barrier function and an inflammatory immune response to environmental antigens. Modified and reprinted, with permission, from the *Annual Review of Nutrition*, Volume 26 © 2006 by Annual Reviews, www.annualreviews.org.


Microbes
Food Proteins/Peptides
Intestinal lumen
Microbial Components / Products
Gut Immune System
APC
MHCI
MHCII
TCR
TCR
CD4+
Th cell
Treg cell
↑Th1 cytokines
Circulation
Pancreas
Immune cell infiltrate (insulitis)
Islet
beta-cell death
beta-cells

The present data suggest that early exposure to wheat precipitates diabetes autoimmunity in a subset of individuals. Information about the role of dietary factors is useful for the development of policies such as the appropriate age of introduction of solid foods. Potential therapies suggested by this work include the avoidance of high risk foods or the consumption of varieties which lack harmful components by individuals with genetic predisposition for diabetes. Early administration of dietary antigen could also be used as a treatment to establish oral tolerance to potentially harmful components (62). Gut microbes can modify diabetes outcome (Figures 5-1 and 5-2; 190, 202). Interventions that alter gut microflora, such as probiotics, oral antibiotics, and dietary protein source, could be used to prevent or treat diabetes (2, 190, 227). However, these seemingly harmless interventions must be considered with caution. Postponed introduction of dietary wheat along with a doubling of the amount of gluten in infant formula in Sweden was associated with a three-fold increase in the incidence of celiac disease (228). Further research is required to clarify the identity and the role of diabetes-promoting cereal proteins in individuals with autoimmune disease susceptibility.

Future Directions

Despite the discovery of insulin as a treatment for diabetes by Banting, Best, Collip, Campbell, and Fletcher in 1922 (229), the pathogenesis of this multifactorial disease remains unclear. The identification of a population of wheat-specific Th1 cells in the gut of some individuals prone to develop diabetes makes it tempting to speculate that these cells could play a role in the Th1-biased insulitis which results in T1D. It remains to be determined whether these cells can migrate to the pancreas and if they participate in T1D pathogenesis.

In order to develop preventive treatments, diabetes-promoting environmental antigens must be identified and their role in T1D pathogenesis must be characterized. Focus should be placed on understanding how diabetes-related dietary proteins, such as wheat, interact with the gut immune system and how this process affects islet biology. Determining how environmental triggers control diabetes expression, will aid with the development of treatments to prevent the immune response against beta-cells.

CONCLUSIONS

The ratio of T-bet to GATA-3 transcription factor expression was validated as a useful method for measuring Th1/Th2 inflammatory cell status in whole tissues (104, 126). GATA-3 was more important than T-bet for maintenance of T cell differentiation. This method identified a pro-inflammatory Th1 state in the gut and associated lymphoid tissues of BBdp rats, which have genetic susceptibility for type 1 diabetes (109, 138). This Th1 bias developed as a result of a relative deficit of the Th2-specific transcription factor GATA-3. A cereal-based diet promoted Th1 cytokine expression in the gut and associated mesenteric lymph nodes of BBdp rats (109, 138). It was also associated with the activation of wheat protein-specific $CD4^+$ T cells, possibly due to a lack of regulatory T cells. In confirmation of the results obtained in rats, similar Th1 immune reactivity to wheat proteins was detected in a subset of patients with T1D and without clinical signs of CD (154). The effect of diet was tested in the Komeda diabetes-prone (KDP) rat. A hydrolyzed casein diet, which reduces T1D incidence in BBdp rats and NOD mice, did not have a similar effect in KDP rats (186). This indicated that diabetes resulting from the unique immune defect in this model is not modifiable by dietary proteins. A mainly wheat-based cereal diet consistently promotes diabetes in BBdp rats. This modification still occurred in the absence of microbes. In the absence of cereal antigens, a diabetes-promoting effect of microbes was revealed. Both the cereal-containing diet and microbes had Th1 cytokine-promoting trends in the gut-associated lymphoid tissues.

Evidence is accumulating that the gut is abnormally inflamed and permeable in some patients with T1D (83, 90) and in animal models of this disease (73, 84, 85, 88, 89). Increased permeability has been directly linked with T1D risk in diabetes-prone rats (87). It

is also clear in animal models, and is becoming evident in patients, that diet can modify diabetes autoimmunity in the pancreas (57, 75, 76, 215). My results provide a strong indication that gut function and gut immune status is abnormal in BBdp rats and in patients with T1D (109, 138, 153). The germ-free study results demonstrate that the major environmental influence on BBdp rat diabetes is diet. Wheat is highly immunogenic in BBdp rats and in T1D patients (109, 138, 153, 154). Therefore we are left with a strong inference that gut dysfunction occurs early in BBdp rats, may have a parallel in patients, and could be a crucial component of T1D pathogenesis (2).

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CONTRIBUTIONS OF COLLABORATORS

Dr. Haiping Wang contributed to the design of the experiment presented in Figures 1-1. Dr. Habiba Chakir designed the experiments, I contributed equally to performing the cell culture, and I performed the analysis for the experimental results presented in Figures 1-2, 1-5, 2-3, 2-4 and 3-1. She also contributed to the cell culture for Figure 1-1 and performed the analysis for Figure 2-5. I thank Majid Mojibian for his important contribution towards establishing the human immunological assays (Chapter 3). I thank Gen-Sheng Wang for instruction on how to score pancreas features (Chapter 4). I thank Dr. Alexander Strom and Ms. Karen Powell for conceptual and artistic assistance with the summary model (Figure 6-1). I am grateful to Jennifer Crookshank, Ahmed Zafer, and Elliott Faller for technical assistance with PCR analyses. I thank S Popovic, D Parks, C Gardell, J Souligny, D Patry, M Kalmokof, and S Brooks from Health Canada, W White and J Goyer from Charles River Laboratories, and J Crookshank from the Scott lab for assisting with the germ-free study (Figure 5-1). In addition, I thank Jocelyn Souligny and Dominique Patry for maintaining the BB rat and KDP rat colonies. I am grateful to Dr. W White at Charles River Laboratories for serology testing of viral antibody levels. I thank Caroline Vergette at the Ottawa Health Research Institute for performing the MoFlo cell sorting.

APPENDICES

APPENDIX 1 – CELL CULTURE CYTOKINES AND ANTIBODIES

REAGENT NAME	ANTIBODY CLONE	COMPANY
anti-rat CD3 antibody	G4.18	BD Canada / Pharmingen, Oakville, ON
anti-rat CD28 antibody	JJ319	BD Canada / Pharmingen, Oakville, ON
Mouse anti-rat IL-4 monoclonal antibody	OX-81	BD Canada / Pharmingen, Oakville, ON
Mouse anti-rat IFN-γ monoclonal antibody	DB-1	Invitrogen Canada, Burlington, ON
Recombinant human IL-12 protein		R&D Systems, Minneapolis, MN
Recombinant rat IL-4 protein		BD Canada / Pharmingen, Oakville, ON
Recombinant human IL-2 protein		Dr. Craig Reynolds, National Cancer Institute, Bethesda, MD
Recombinant human IL-7 protein		PeproTech, Rocky Hill, NJ

APPENDIX 2 – HUMAN FLOW CYTOMETRY ANTIBODIES

ANTIBODY NAME	ANTIBODY CLONE	COMPANY
anti-human CD3-ECD	UCHT1	Beckman Coulter / Immunotech, Mississauga, ON
rat anti-human β7 integrin-PE	F1B504	BD Canada / Pharmingen, Oakville, ON
anti-human CD19-PE	J4.119	Beckman Coulter / Immunotech, Mississauga, ON
anti-human CD83-PE	HB15a	Beckman Coulter / Immunotech, Mississauga, ON
mouse anti-human CD3-sprd	UCHT1	SouthernBiotech, Birmingham, AL
anti-human CD4-FITC	T4-FITC	Beckman Coulter / Immunotech, Mississauga, ON
anti-human CD8-PE	T8-RDI	Beckman Coulter / Immunotech, Mississauga, ON
anti-human CD56-PE	B159	BD Canada, Oakville, ON

APPENDIX 3 – RAT FLOW CYTOMETRY ANTIBODIES

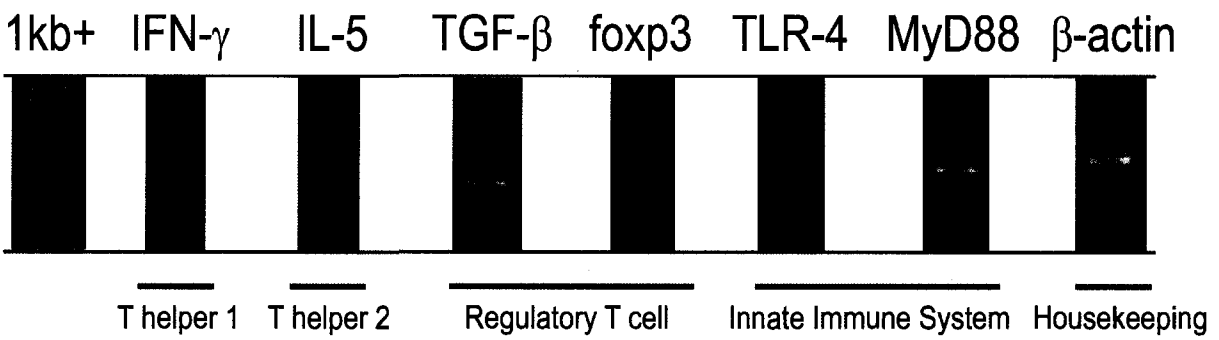
ANTIBODY NAME	ANTIBODY CLONE	COMPANY
mouse anti-rat αβTCR-FITC	R73	Beckman Coulter/Immunotech, Mississauga, ON
anti-rat αβTCR-PE	R73	Cedarlane, Burlington, ON
mouse anti-rat CD3-FITC	G4.18	BD Canada / Pharmingen, Oakville, ON
mouse anti-rat CD4-cy-chrome	OX-35	BD Canada / Pharmingen, Oakville, ON
mouse anti-rat CD8b-FITC	341	BD Canada / Pharmingen, Oakville, ON
mouse anti-rat CD45RA-PE	OX-33	Beckman Coulter/Immunotech, Mississauga, ON
mouse anti-rat macrophage-PE	HIS-36	BD Canada / Pharmingen, Oakville, ON
mouse anti-rat CD25-PE	OX-39	AbD Serotec, Raleigh, NC
mouse anti-rat OX62-biotin	MRC OX-62	AbD Serotec, Raleigh, NC
Streptavidin-ECD	-	Beckman Coulter/Immunotech, Mississauga, ON

APPENDIX 4 – PCR PRIMERS

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Annealing Temp. (°C)	Product Size (bp)	Ref.
rat T-bet	AACCAGTATCCTGTTCCCAGC	TGTCGCCACTGGAAGGATAG	58	436	-
rat GATA-3	CTCTCCTTTGCTCACCTTTTC	AAGAGATGCGGACTGGAGTG	58	619	-
rat IFN-γ	CGTCTTGGTTTTGCAGCTC	ACTCCTTTTCCTCTTCCTTA	53	461	-
rat IL-4	CTTGCTGTCACCCTGTTC	CATGGAAGTGCAGGACTGCA	63	420	-
rat IL-10	TGCCTTCAGTCAAGTGAAGACT	AAACTCATTCATGGCCTTGTA	55	346	-
rat TGF-β	CTTCAGCTCCACAGAGAAGAACTGC	CACGATCATGTTGGACAACTGCTCC	62	298	(39)
rat IL-5	TGCTTCTGTGCTTGAACGTTCTAAC	TTCTCTTTTTGTCCGTCAATGTATTTC	58	298	(230)
rat TLR-4	CCCTGCATAGAGGTACTTCC	CCATGCCATGCCTTGTCTTC	58	218	-
rat MyD88	CTTGCACCAAGTCCTGGTTC	AGACAGGATGCCACCTCAAG	58	268	-
rat foxp3	CCCAGGAAAGACAGCAACCTT	CTGCTTGGCAGTGCTTGAGAA	60	133	(231)
rat β-actin	CCAGCCTTCCTTCCTGGGTA	CTAGAAGCATTTGCGGTGCA	55	343	-
human IFN-γ	TGTTACTGCCAGGACCCAT	GCGTTGGACATTCAAGTCAG	63	330	(127)
human IL-4	ACAAGTGCGATATCACCTTAC	CAACGTACTCTGGTTGGCT	63	326	(127)
human β-actin	ACACTGTGCCCATCTACGAGGGG	ATGATGGAGTTGAAGGTAGTTTCGTGGAT	55	462	(127)

Figure 6-2. RT-PCR products from selected optimized primers

Gene expression of IFN-γ, IL-5, TGF-β, foxp3, TLR-4, MyD88 and β-actin measured by RT-PCR in total RNA pooled from the MLN tissue of four GF and four SPF BBdp rats. PCR was performed at the optimal cycle number which was selected in the region of linearity between cycle number and PCR product intensity. PCR products were separated by electrophoresis in 1.5 % agarose gel along side the 1kb Plus DNA ladder to confirm the correct product size. Data are representative of triplicate samples.


1kb+
IFN-γ
IL-5
TGF-β
foxp3
TLR-4
MyD88
β-actin
T helper 1
T helper 2
Regulatory T cell
Innate Immune System
Housekeeping

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David Lefebvre

Ottawa Health Research Institute

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ETHICAL APPROVAL

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Conseil d'éthique en recherches
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Fax No. ~ 761-4311
http://www.ohri.ca/ohreb/

Monday, September 18, 2006

Dr. Fraser Scott
Molecular Medicine Program
Autoimmune Disease Group, OHRI
Ottawa Hospital - General Campus
501 Smyth Road
Ottawa, ON K1H 8L6

Dear Dr. Scott:

Re: Protocol # 2003029-01H Screening the Wheat Proteome for Proteins Related to Human Type 1 Diabetes - JDRF Grant 1-2004-283

Thank you for the protocol amendment dated August 30, 2006. It is approved.

Ethical approval remains in effect until June 16, 2007.

Yours sincerely,

Raphael Saginur, M.D.
Chairman
Ottawa Hospital Research Ethics Board

/rf

CURRICULUM VITAE

David E. Lefebvre

TRAINING

May 2002 – Feb 2007 University of Ottawa, Ottawa, ON
Doctorate of Science (Ph.D.) Biochemistry
- Thesis: Inflammatory immune reactivity to cereal proteins in type 1 diabetes

Sept. 1998-April 2002 University of Ottawa, Ottawa, ON
Baccalaureate in Science Honours Biochemistry (Magna Cum Laude)
- Thesis: T-bet/GATA-3 gene expression as a measure of Th1/Th2 cell status in the diabetes-prone BB rat

WORK EXPERIENCE

2002 – 2007 Ottawa Health Research Institute
PhD Biochemistry Graduate Student, Identification of reactivity to cereal in type 1 diabetes

2006 Faculty of Science, University of Ottawa
Midterm Exam Proctor, Cellular regulation and control

2006 Faculty of Science, University of Ottawa
Teaching Assistant, Biochemistry laboratory I

2005 Faculty of Science, University of Ottawa
Teaching Assistant, Molecular biology laboratory

2001 – 2002 Ottawa Health Research Institute
Research Assistant, Cytokine gene expression analysis

1998 – 2000 Molecular Genetics Laboratory, CHEO Research Institute
Research Assistant, Molecular biology research for the study of muscular dystrophy

PEER-REVIEWED PUBLICATIONS

Lefebvre DE, Powell KL, Strom A, Scott FW. Dietary protein antigens as environmental modifiers of type 1 diabetes mellitus. **Annual Review of Nutrition**. 2006;26:175-202.

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. **Diabetes Care**. 2006 May;29(5):1108-1110.

Chakir H*, **Lefebvre DE***, Wang H, Caraher E, Scott FW. Wheat protein-induced pro-inflammatory T helper 1 bias in mesenteric lymph nodes of young diabetes-prone rats. **Diabetologia**. 2005 Aug;48(8):1576-84. *** Authors contributed equally**.

Chakir H, Wang H, **Lefebvre DE**, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. **Journal of Immunological Methods**. 2003 Jul;278(1-2):157-69.

MANUSCRIPTS IN PREPARATION

Lefebvre DE, Wang G-S, Scott FW. Lack of a protective effect of dietary hydrolyzed casein on type 1 diabetes in the Komeda diabetes-prone rat.

Lefebvre DE, Wang G-S, Patrick C, Crookshank J, Popovic S, Parks D, Souligny J, Gardell C, Brooks S, Kalmokof M, Scott FW. Parsing the role of microbes and diet in the development of autoimmune diabetes.

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

09/2006 First Nordic Meeting on Genetics and Pathogenesis of Immunological Diseases, Helsinki, Finland
Abstract: Wheat Protein Immune Reactivity in Human Type 1 Diabetes Linked to HLA-DR4. Mojibian M, Chakir H, **Lefebvre DE**, Scott FW.

06/2005 American Diabetes Association 65th Scientific Sessions, San Diego, CA
Abstract: T cell reactivity to wheat proteins in human type 1 diabetes. Mojibian M, **Lefebvre DE**, Chakir H, Scott FW. **Diabetes** 54: A79-A79 Suppl. 1 2005.

04/2005 Experimental Biology 2005, San Diego, CA
Poster Presentation: Type 1 diabetes frequency is not modified by a hydrolyzed casein diet in the Komeda diabetes-prone rat. **Lefebvre DE**, Scott FW. **FASEB Journal** 19(4):A83-A84 Part 1 Suppl. S, Mar. 4, 2005.

12/2004 The BB Rat Turns Thirty: Lessons and Future Directions International Symposium, Ottawa, ON
Poster Presentation: Wheat protein-induced inflammatory Th1 bias in gut-associated lymphoid tissues of diabetes-prone BB rats. **Lefebvre DE**, Chakir H, Caraher E, Scott FW.

11/2004 Ottawa Health Research Institute Research Day Diabetes and Obesity Symposium, Ottawa, ON
Oral Presentation: Wheat protein-induced inflammatory Th1 bias in gut-associated lymphoid tissues of the diabetes-prone BB rat.

04/2004 Experimental Biology 2004, Washington, DC
Poster Presentation: Wheat gluten-induced pro-inflammatory Th1 bias in mesenteric lymph nodes of diabetes-prone BB rat. **Lefebvre DE**, Chakir H, Scott FW. **FASEB Journal** 18(4):A496-A496 Suppl. S, Mar. 23 2004.

04/2004 Experimental Biology 2004 - American Society for Nutritional Sciences Procter and Gamble Graduate Student Oral Competition, Washington, DC
Oral Presentation: Wheat gluten-induced pro-inflammatory Th1 bias in mesenteric lymph nodes of diabetes-prone BB rat.

06/2002 11th International Congress of Mucosal Immunology, Orlando, FL
Oral and Poster Presentations: T-bet/GATA-3 gene expression as a measure of Th1/Th2 status in mesenteric lymph nodes and Peyer's patches of BB rats. Wang H, **Lefebvre DE**, Scott FW. **Society for Mucosal Immunology - Mucosal Immunology Update** 2002; 10(2 and 3):6021.

HONOURS AND AWARDS

2003 - 2006 Ontario Ministry of Training, Colleges and Universities
Ontario Graduate Scholarship ($15,000/year)

2002 - 2007 University of Ottawa
Faculty of Graduate Studies Excellence/Admission Scholarship ($5430- $5535/year)

2004 American Society for Nutritional Sciences, Experimental Biology 2004
Nutrition Immunology Graduate Student Poster Competition Award ($250 US)

2004 American Society for Nutritional Sciences, Experimental Biology 2004
Procter&Gamble Graduate Student Research Abstract Competition Travel Award ($750 US)

2003 - 2004 University of Ottawa
Strategic Areas of Development Scholarship ($6000)

2003 University of Ottawa
Biochemistry Graduate Student Poster Day - First place M.Sc. poster ($125)

2002 11th International Congress of Mucosal Immunology, Orlando, FL
Young Investigator Award ($800 US)

2002 University of Ottawa
Biochemistry Program Entrance Award ($4000)

2000 - 2002 University of Ottawa
Faculty of Science Dean's Honour List

2001 - 2002 University of Ottawa
Faculty of Science Merit Scholarship ($500)

1998 - 1999 University of Ottawa
Undergraduate Admission Scholarship ($4000)