CEREAL INDUCED AUTOIMMUNE DIABETES IS ASSOCIATED WITH SMALL INTESTINAL INFLAMMATION, DOWNREGULATED ANTI-INFLAMMATORY INNATE IMMUNITY AND IMPAIRED PANCREATIC HOMEOSTASIS

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ABSTRACT

Background: Intestinal inflammation elicited by environmental determinants including dietary proteins and microbes is implicated in type 1 diabetes (T1D) pathogenesis. Also, intrinsic pancreatic abnormalities could precede classic insulitis, contributing to T1D. ***Materials and Methods:*** Spontaneous rat T1D models were used for *in situ* analyses of gut and pancreas to explore novel disease pathways using immunohistochemistry and detailed morphometry, gene expression studies, and molecular screening analyses. ***Results:*** In BBdp rats, feeding a cereal diet stimulated T1D under germ-free or specific pathogen-free (SPF) conditions compared with a protective hydrolyzed casein (HC) diet. Cereal-induced T1D was paralleled by increased gut T cell infiltration and T_H1-associated pro-inflammatory transcription. HC-fed rats displayed an increased number of anti-inflammatory $CD163^+$ M2 macrophages compared with cereal-fed rats. Cereal-associated promotion of T1D in Lewis diabetes-prone (LEW-DP) rats, a different rat model, similarly featured gut T cell infiltration in conjunction with decreased immunoregulation. The *Camp* gene was induced in diet-protected HC-fed BBdp rats. *Camp* encodes the cathelicidin antimicrobial peptide (CAMP), a pleiotropic immunomodulatory host defence factor. Intestinal CAMP was enriched in $CD163^+$ M2 macrophages and could represent a novel marker of these tolerogenic innate immune cells. CAMP expression was also discovered in pancreatic lymph nodes (PLN) and islets, indicating a novel role for this factor in target tissue homeostasis. There was a positive correlation between pancreatic CAMP and total islet number. Also, islet-associated $CAMP^+$ cells were increased in rats with islet inflammation, suggesting upregulation in parallel with insulitis. Exogenous CAMP/LL-37 injections increased the abundance of T1D-protective probiotic bacteria and promoted islet neogenesis in BBdp rats. A prospective partial pancreatectomy (PPx) study was performed to obtain pre-diabetic pancreas biopsies from

pre-insulitic BBdp rats. The number of endothelium-associated $CD68^+$ macrophages was increased in pre-diabetic pancreata, indicating that perivascular inflammation was an early lesion in the animals. In addition, pre-diabetic pancreata featured enhanced regenerative *Reg3a* and *Reg3b* gene expression, indicating abnormal islet expansion preceding insulitis. ***Conclusions:*** Small intestinal inflammation paired with deficits in local immunoregulation parallels T1D development. CAMP represents a novel factor in T1D that could have several pleiotropic functions including regulation of commensal microbes, intestinal homeostasis, and pancreatic homeostasis. In addition, target tissue abnormalities precede insulitis and T1D. This research focused on the integrative biology of T1D pathogenesis in spontaneous rat models. This work provides a novel working model that incorporates key roles for gut lumen antigens, intestinal immunity, and the role of islets and altered regenerative capacity in T1D. This research could lead to new therapeutic opportunities for T1D treatment.

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LIST OF ABBREVIATIONS

APC: Antigen-presenting cell
BBc: BioBreeding control rat; diabetes-resistant (Ottawa strain)
BBdp: BioBreeding diabetes-prone rat (Ottawa strain)
BB-DP: BioBreeding diabetes-prone rat (Worcester strain)
BB-DR: BioBreeding control rat; diabetes-resistant (Worcester strain)
DC: Dendritic cell
DSS: Dextran sodium sulphate
EAE: Experimental autoimmune encephalomyelitis
EIC: Insulin^{+} extra-islet cluster
FOXP3: Forkhead/winged homeobox 3
GWAS: Genome-wide association study
HLA: Human leukocyte antigen
IFN-γ: Interferon-gamma
IDDM: Insulin-dependent diabetes mellitus (human T1D genetic risk locus designation)
Iddm: Rat T1D genetic risk locus designation; **Idd** - mouse locus.
IEL: Intra-epithelial lymphocyte
iNOS: Inducible nitric oxide synthase
IPEX: Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome
KRV: Kilham rat virus
LEW-DP: LEW.1AR1/Ztm-*iddm* diabetes-prone rat
LPL: Lamina propria lymphocyte
MAdCAM-1: Mucosal vascular addressin cell adhesion molecule 1
MHC: Major histocompatibility complex
MLN: Mesenteric lymph node
NF-κB: Nuclear factor kappa-B
NO: Nitric oxide
NOD: Nonobese diabetic mouse; diabetes-prone mouse
OVA: Ovalbumin
PLN: Pancreatic lymph node
SCFA: Short chain fatty acid
SPF: Specific pathogen-free
STZ: Streptozotocin
STAT: Signal transducer and activator of transcription
T1D: Type 1 diabetes mellitus
TLR: Toll-like receptor
TNF-α: Tumour necrosis factors alpha
T_{reg}: Regulatory T cell

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CHAPTER 1. Introduction

Overview of thesis

The research focus was to investigate the role of environment on development of spontaneous autoimmune type 1 diabetes (T1D) in rat models *in vivo* to gain a better understanding of pathogenesis and identify novel therapeutic opportunities. In the present *Chapter*, basic concepts relating to T1D are briefly reviewed, including clinical features, genetic and epidemiological aspects, rodent models, environmental influence, and a description of the prevailing model of T1D pathogenesis. Emphasis is placed on the role of the gastrointestinal tract as a key organ integrating environmental signals and the immune system, which could be key to understanding this complex chronic disease. *Chapter 2* is a description of the materials and methods used to perform the studies described in *Chapters 3-6*. The roles of environment and intestinal inflammation in T1D development are investigated in two different rat models: The influence of diet and microbes in diabetes-prone BioBreeding (BBdp) rats is explored in *Chapter 3* and the role of diet is investigated in diabetes-prone Lewis rats in *Chapter 4*. In *Chapter 5*, a screening study in the intestine of diet-protected BBdp rats forms the basis for identification and characterization of a host defense factor (cathelicidin antimicrobial peptide) implicated in T1D protection. In *Chapter 6*, early pancreatic abnormalities are investigated in BBdp rats, which could represent novel aspects of T1D pathogenesis. The work is summarized in *Chapter 7*.

Type 1 Diabetes

General introduction and clinical features: Type 1(A) diabetes (T1D) is a severe chronic disease caused by loss of immune regulation and autoimmune destruction of insulin-producing pancreatic beta (β)-cells (1-7). T1D is a complex polygenic disease, requiring

interplay between host genetics and environment (8-11). Lifelong dependence on exogenous insulin injections is necessary for survival, although this does not represent a cure. Debilitating secondary complications can eventually arise, including renal failure, stroke, blindness, heart disease, and amputation, all of which contribute to increased morbidity and decreased life expectancy (12).

Genetic susceptibility: The human leukocyte antigen (HLA) region on chromosome 6, known as *IDDM1*, is the most important locus, conferring 40-60% of genetic risk (4; 13). Only a small percentage of individuals develop T1D despite a much larger percentage possessing susceptible HLA haplotypes, indicating the presence of additional risk factors (2). More recently, the traditional HLA risk genes have been less frequently associated with new cases, implying an even greater role for environment than previously appreciated (14). Non-HLA-associated susceptibility loci identified by genome-wide association studies (GWAS) account for only a small proportion of disease heritability, with the most prevalent polymorphisms accounting for less than 30% of heritable risk (15; 16). Such loci include the variable number of tandem repeats in the promoter upstream of the insulin (*INS*) gene on chromosome 11 (*IDDM2*), as well as the immune-associated locus *PTPN22* (LYP) on chromosome 1, *CTLA4* (cytotoxic T lymphocyte associated-4; *IDDM12*), and *IL2RA* (CD25), although the contribution of each locus is relatively low (7). Interestingly, approximately half of T1D-associated risk genes encode products expressed in β-cells (17), suggesting intrinsic involvement of the target tissue in establishing susceptibility to autoimmunity.

Epidemiology: Over 5 million individuals worldwide have T1D (18). In Western countries, T1D prevalence is ~0.4% (2) and incidence has been dramatically rising (5; 19), increasing ~3% per year among children and adolescents according to the International Diabetes Federation (www.idf.org) (20). Lifetime risk in the general population has surpassed 1% in North America and Europe (8; 20). In Europe, children between one and five years of age represent the age demographic with the most rapidly increasing incidence (21). In 85% of newly-diagnosed patients, there is no record of familial T1D (22; 23). Concordance rates among monozygotic twins are substantially less than 100% (24; 25), even when discordant twins are followed for up to 40 years after the initial twin develops T1D (13). In monozygotic and dizygotic twins, concordance is approximately 30-50% (26) and 10% (7; 27), respectively. The differences in disease expression among twins are likely attributable to a combination of non-germ-line encoded (somatic) variations and environmental influence (13). Environmental factors could operate as triggers, accelerators, or inhibitors of β-cell autoimmunity. Autoantibodies indicative of active islet autoimmunity are frequently detected in young children that progress to T1D, suggesting a critical role for environment in modulating disease at early stages of diabetogenesis (1; 7; 28). T1D prevention or therapy will require an increased understanding of environmental disease modifiers and the implementation of preventive strategies or combination therapies that modulate inflammation and promote β-cell replenishment (21; 29).

Animal models of spontaneous T1D

Nonobese diabetic (NOD) mice: The NOD mouse has been available for over thirty years and is currently the most widely used rodent model of T1D. As in humans, T1D in NOD mice occurs spontaneously and is under polygenic control (30). Genetic susceptibility is conferred

by several risk loci including MHC class II genes, namely the $H2^{g7}$ haplotype (4). Unlike humans, incidence is markedly increased in females (~80%) compared with males (~10-30%) (2). Immunosuppression inhibits disease development and environmental factors such as diet have an influence on incidence (31-33).

BioBreeding diabetes-prone (BBdp) rats: BBdp rats spontaneously develop a polygenic (34) form of T1D equally in males and females that is characterized by a T_H1-biased destruction of β-cells (35-38). Genetic risk is conferred by *RT1*^{u} genes from the major histocompatibility (MHC) complex (class II) (*Iddm2*) (39). In addition, a mutation on chromosome 4 (*Gimap5*; *Iddm1*) causes a pronounced T cell lymphopenia that is required for islet autoimmunity (4). Islet inflammation (insulitis) becomes evident during adolescence (~30-50 d) and overt T1D occurs thereafter (~60-150 d) (2). Metabolic outcomes of overt T1D in these animals include hyperglycemia, weight loss, and reduced plasma insulin concentration (39), similar to human disease. Ultimately, untreated BBdp rats succumb to T1D due to hyperglycemia and ketoacidosis unless supplied with exogenous insulin (39). Immunosuppression inhibits development of disease and environmental factors such as diet have a strong influence on T1D incidence, consistent with NOD mice (2; 32; 38; 40; 41).

LEW.1AR1/Ztm-iddm (LEW-DP) rats: The LEW-DP rat provides an interesting alternative to the traditional rodent models of spontaneous T1D (38; 42). Consistent with BBdp rats, susceptibility is conferred by *RT1*^{u} genes of the MHC class II locus (38; 42), although it is unclear yet whether this model is polygenic or oligogenic. Unlike the BBdp rat, there is no pronounced lymphopenia and unlike the NOD mouse, there is no sex-bias with respect to

incidence (42). Consistent with BBdp rats and NOD mice, insulitis in LEW-DP rats is characterized by infiltration of macrophages and T cells leading to β-cell destruction (43).

T1D pathogenesis

Role of gastrointestinal tract: An emerging paradigm for the development of autoimmune diseases, including T1D, involves three distinct components (10). The primary component is genetic susceptibility of the host immune system, predisposed to abnormally respond to environmental antigens encountered in the gut; the second component is the requirement for the host to be exposed to the provocative environmental antigen; the third component is the requirement of the provocative environmental antigen to translocate across the gut epithelium and gain direct access to the underlying impaired host immune system (10). Gut-originating inflammation could then disseminate, culminating in the activation of an adaptive immune response with specificity for pancreatic β-cells. In BBdp rats, intestinal inflammation is reflected by increased expression of T_H1 cytokine IFN-γ in the gut-draining mesenteric lymph nodes (MLN) (36; 44), which are the body's largest lymph nodes critical for oral tolerance (45). An increased *Tbet*/*Gata3* mRNA transcription factor ratio, reflective of T_H1 bias, was also reported in the MLN of BBdp compared with BBc rats (36). Both MLN and spleen of BBdp rats displayed a decreased percentage of T_{reg} compared with BBc rats, indicating impaired adaptive immune regulation (36). This finding was consistent with another report, in which BB-DP rats (Worcester strain) displayed a decreased percentage of regulatory forkhead/winged-helix Box P3 $(Foxp3)^+$ T_{reg} in MLN compared with control (diabetes-resistant) BB-DR rats (46). Also, $Foxp3^+$ T_{reg} from BB-DP rats were defective in suppressing the proliferation of BB-DR effector T cells, indicating impaired regulatory

function in the diabetes-prone strain (46). Thus, pro-inflammatory T_H1-biased MLN immune activation occurs in parallel with decreased immune regulation in BBdp rats. It was also demonstrated that adoptive transfer of MLN cells from young (3-week old) NOD mice into NOD/*scid* recipients induced insulitis and T1D (47). A key question is whether immune inflammation in MLN is also reflected at the small intestinal mucosa (e.g. epithelium and lamina propria), where interaction with environmental antigens occurs upstream of MLN.

Small intestinal leakiness: The intestinal epithelium is a protective cellular layer that separates the body's tissues from the environmental contents of the gut lumen. In addition to providing a structural barrier, the epithelium is a major controller of immunological and nutritional homeostasis. Intestinal leakiness and gut barrier impairment are emerging as important contributors to T1D (9; 10; 48; 49). Impaired gut barrier function preceding T1D has been described in humans and impaired regulation of gut permeability could form the physiological basis for disease susceptibility (50). BBdp rats display increased small intestinal permeability that precedes onset of insulitis and overt T1D (51-53). Epithelial apical tight junctions are complexes that regulate intestinal permeability in the small intestine (54; 55). Zonulin/pre-haptoglobin2 (zonulin/pre-HP2) (56) is currently the only characterized physiological modulator of tight junctions and impairment of this system is postulated to be a key factor promoting chronic intestinal inflammation and autoimmunity (57). The natural physiological role of zonulin/pre-HP2 is speculated to involve protection of the small intestinal mucosa from excessive microbial colonization (10). In BBdp rats, abnormally increased concentration of zonulin/pre-HP2 was observed in parallel with the progression of insulitis (58). Treatment of BBdp rats with an inhibitor of the zonulin/pre-HP2 receptor (AT-1001) prevented intestinal leakiness and inhibited T1D development (56; 58). Interestingly,

increased serum concentration of zonulin/pre-HP2 has been reported in a subset of human T1D patients and their first-degree relatives (59), consistent with gut leakiness contributing to human T1D. We hypothesize that excessive translocation of environmental antigens in the gut of diabetes-prone subjects occurs in parallel with stimulation of pro-inflammatory intestinal immune responses that ultimately activate β-cell autoimmunity.

Small intestinal inflammation: The small intestine could play a major immunological role in T1D pathogenesis. A constitutive histological enteropathy, featuring small intestinal villus reduction and crypt hyperplasia, is present in BBdp rats; the enteropathy precedes T1D and is absent from control BBc rats (44). A similar form of enteropathy has been described in NOD mice (60). In a seminal study, Turley *et al*. demonstrated that ovalbumin (OVA)-specific T cells proliferated in pancreatic lymph nodes (PLN) upon oral administration of OVA protein (61). This experiment directly demonstrated a functional immunological link between oral antigen exposure and immune activation in PLN (61). Treatment of NOD mice with dextran sodium sulphate (DSS), a chemical agent that damages the intestinal mucosa, stimulated T cell proliferation in both MLN and PLN; in addition, DSS exposure promoted insulitis (61). PLN are a critical source of β-cell-reactive immune cells, as their surgical removal at 3 weeks (weaning) in NOD mice inhibited insulitis and T1D (62). Furthermore, adoptive transfer of NOD-derived PLN cells induces T1D in NOD/*scid* mice (47). Colonic infection of NOD mice with the enteric bacterium *Citrobacter rodentium*, a pathogen that disrupts the epithelial barrier, accelerated insulitis and T1D (63), consistent with results from Turley *et al*. using DSS (61). The chronically inflamed gut possibly represents the primary source of activated β-cell-specific autoreactive immune cells (2). Consistent with this notion, gut-

originating T cells expressing the gut-homing integrin $\alpha_4\beta_7$ have been described as a population of early-infiltrating immune cells in the pancreata of young NOD mice (64; 65). The mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), the ligand for $\alpha_4\beta_7$ integrin, has likewise been shown to be induced on islet vasculature of NOD mice (66). Treatment of NOD mice with antibodies directed against either integrin β_7 or MAdCAM-1 inhibited T1D (67). Thus, a large body of evidence supports the involvement of small intestinal immune responses and T1D development.

Environmental modulation of gut immunity and T1D

Dietary influence on T1D in animal models: When exposed to innocuous dietary factors in the gut, the mucosal immune system remains locally and systemically non-responsive via a process known as "oral tolerance", which is essential for both mucosal and systemic immune homeostasis (45). Breakdown of this intricate regulatory network can result in loss of intestinal homeostasis and development of chronic inflammatory syndromes, including allergy and autoimmunity (45). Induction of oral tolerance is achieved by various anti-inflammatory immune cell types (T_{reg}, tolerogenic DC, and anti-inflammatory M2 macrophages) and involves distinct processes in the inductive sites of the gut immune system, such as the MLN, as well as the effector sites, comprised of epithelium and lamina propria (45). In BBdp rats, weaning onto a cereal diet promotes T1D compared with low-antigen diets (41). Feeding BBdp rats a protective hydrolyzed casein (HC) diet improved intestinal barrier integrity, as demonstrated by decreased permeability and lower concentration of circulating zonulin/pre-HP2 (52). Also, early oral exposure of neonatal BBdp rats to dietary antigens from a cereal-based diet decreased expression of T_H1 cytokine

IFN-γ in the gut, likely a reflection of oral tolerance induction (68). Interestingly, MLN-derived T_H1 cells from BBdp rats proliferated *in vitro* upon exposure to wheat peptides; also, protective HC feeding decreased the number of IFN-γ^+ T cells in MLN, indicating dietary modulation of gut inflammation (36). In the NOD mouse, cereal feeding promoted T1D compared with low-antigen diets, such as gluten-free or modified gluten-free formulas (31; 32; 69). Consistent with these reports, avoidance of wheat exposure entirely, weaning onto a wheat protein-free diet, and delayed exposure to wheat also afforded protection (70; 71). In spontaneous animal models, diabetes-modifying diets potentially modulate T1D by beneficial alteration of a combination of intestinal parameters, such as barrier integrity, inflammatory status, and microbiota composition. The proximal jejunum is the primary site of protein digestion and absorption in the gastrointestinal tract (72). Protein hydrolysis in the jejunum by the combined enzymatic actions of pepsin and trypsin as well as brush border peptidase mostly results in short peptides and free amino acids, which are subsequently internalized by enterocytes using active transport mechanisms (72). Because proteins ultimately drive the activation of adaptive immunity, the jejunum is a key segment of the gastrointestinal tract for exploration of dietary modification of T1D, as this is the location where ingested proteins are most likely to interact with the mucosal immune system.

Dietary influence on T1D in humans: The most intensely investigated dietary factors implicated in human T1D pathogenesis include breastfeeding duration, cow milk protein consumption in early life, and exposure to cereal proteins in early life (2; 73). Infant exposure to complex dietary proteins has been linked to increased T1D risk (74; 75). Early feeding of cow milk proteins has been associated with enhanced T1D risk in rodent models

and humans (2; 76). The controversial relationship between cow milk proteins in infant formulas and T1D development is currently being evaluated in an intervention trial called the "Trial to Reduce IDDM in the Genetically at Risk" (TRIGR) (2). The TRIGR trial represents the largest study of dietary intervention for primary prevention of T1D (2). Weaning onto an HC-based formula (Nutramigen) compared with a cow milk formula decreased signs of β-cell autoimmunity in children (77; 78). Increased risk for islet autoimmunity in susceptible infants has also been associated with the timing of cereal protein introduction (79; 80).

Early childhood illnesses, diet, and subsequent presence of islet autoimmunity were investigated in the "Diabetes Autoimmunity Study in the Young" (DAISY), which is a large prospective study comprising control children and genetically at-risk children with at least one first degree relative with T1D (81). Dietary intake of cereals (wheat or barley) during the first 3 months of life (early) or after 7 months (late) was linked to increased risk of islet autoimmunity (81). Also, a significant interaction between the occurrence of gastrointestinal infections and later development of islet autoimmunity was dependent on timing of exposure to cereals (81). Only among infants exposed to cereals either at early or late time points were gastrointestinal illnesses significantly associated with increased islet autoimmunity at a later time point (81). Thus, gastrointestinal illnesses, which importantly occurred secondary to cereal exposure, were associated with promotion of islet autoimmunity in at-risk infants with impaired immune systems. A human study by our group found that *in vitro* wheat peptide challenge of peripheral blood mononuclear cells (PBMCs) from a large subset of T1D patients stimulated proliferation and a mixed pro-inflammatory cytokine response characterized by production of IFN-γ, TNF-α, IL-17A, and IL-6 (82). However, blockade of

HLA-DR inhibited activation and pro-inflammatory cytokine secretion in peripheral T cells from patients exposed to wheat proteins, demonstrating that the dietary effect was T1D-specific (82).

There is overlapping genetic risk between T1D and celiac disease, a gluten-sensitive enteropathy; the HLA-DQ2 haplotype, which is present in the majority of patients with celiac disease, occurs in approximately 40% of patients with T1D (2). Prevalence of celiac disease is higher in patients with T1D (~1-8%) compared with the general population (<1%) (2; 83). These figures suggest that some aspects of T1D could be shared with celiac disease, with abnormal immune processing of cereal proteins in the gastrointestinal tract potentially playing an important common role in pathogenesis of both syndromes. However, the study by our group demonstrated that abnormal T cell proliferation to wheat peptides was associated with the T1D risk gene HLA-DR4 but was unrelated to the major celiac disease risk gene HLA-DQ2 (82). Our group previously reported the case of a highly wheat-sensitive patient with both T1D and celiac disease who exhibited strong antibody reactivity to a wheat storage globulin (subsequently renamed Glo-3A) with increased $CD3^+$ T cell proliferation upon exposure to wheat gluten (84). The patient's PBMCs produced a high amount of IFN-γ in response to Glo-3A exposure (84). Taken together, the studies demonstrate induction of T_H1-biased inflammation in a subset of cereal-exposed humans with T1D. Thus, examining dietary modulation of gut immunity could provide new insights into T1D pathogenesis as the first encounter of cereal antigens is with the immune system in the gastrointestinal tract.

Microbial influence on gut immunity and T1D: In humans, there are approximately 10-fold more bacterial cells compared with host mammalian cells, containing a metagenome 100-

fold larger than the human genome (85; 86). Despite the large numbers of microbes residing in the mammalian gut, hosts normally co-exist in a mutualistic association with commensal bacteria, without induction of pro-inflammatory immune responses characteristic of infection. Similar to oral tolerance to food antigens, the gut-associated mucosal immune system remains locally hyporesponsive to commensal microbes in a process termed "mucosally-induced tolerance" (45). A key point is that immune tolerance to commensal microbes is different from oral tolerance to food antigens, in that hyporesponsiveness to microbes does not extend systemically beyond the mucosal microenvironment (45). Thus, the influence of diet on tolerogenic aspects of the immune system is potentially more extensive than microbial influence. Microbial association with T1D in BBdp rats has been established. In a study by Roesch *et al*., BB-DP and BB-DR fecal samples were compared and an increased abundance of *Lactobacillus* and *Bifidobacterium* species was detected in the resistant strain (87). When a particular probiotic strain, *Lactobacillus johnsonii* N6.2, was administered colonically to BB-DP rats, T1D was inhibited in association with decreased small intestinal *Ifng* expression (88). In a retrospective study, BBdp rats that progressed to T1D displayed an increased fecal proportion of bacterial species from the *Bacteroides* genus (*Bacteroidetes* phylum) compared with rats that remained asymptomatic (89). When antibiotics were administered to BBdp rats fed a cereal diet, T1D development was inhibited (89). More intriguingly, combining antibiotics with an HC diet completely suppressed T1D (89), demonstrating that microbes and diet jointly interact with the immune system, underscoring the important concept of intestinal "antigenic load" as a T1D risk factor. Consistent with animal models, microbes have also been associated with T1D in humans. *Bacteroidetes* (phylum), *Bacteroidaceae* (family), and *Bacteroides* (genus) were more abundant in autoantibody-positive subjects compared with those that were autoantibody-

negative (90). Bacteria from the *Bacteroides* genus have been previously associated with T1D in both BBdp rats and humans (89; 91). Association of gut bacteria with T1D is consistent with gastrointestinal involvement in pathogenesis (58; 90; 92). Bacteria could influence T1D via modulation of intestinal immune status.

Regulatory immune cell subsets implicated in T1D modulation

A deficit in immune regulation in the gastrointestinal tract could enable gut inflammation and underlie susceptibility to environmental induction of T1D. Regulatory T cells (T_{reg}) and M2 macrophages are both immune populations with anti-inflammatory properties that could modulate intestinal inflammation and decrease T1D risk. Neither population has been investigated in the jejunum of diabetes-prone rats. These populations are briefly described:

Regulatory T cells: $Foxp3^+$ T_{reg} play a central lifelong role in preventing autoimmunity in rodents and humans (1; 93; 94). Foxp3 is a cell lineage-determining master transcription factor governing T_{reg} differentiation and function (93). $Foxp3^+$ T_{reg} suppress the proliferation and function of various immune cells, including effector $CD4^+$ and $CD8^+$ T cells, B cells, DC, macrophages, and natural killer cells (95). Mice with a loss-of-function mutation or deficient in the *Foxp3* gene develop lethal autoimmunity (94). In humans, mutations in the *FOXP3* gene result in the "Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX)" syndrome, featuring intestinal inflammation and islet autoimmunity (96). Abnormalities in $Foxp3^+$ T_{reg} have been reported in T1D patients (97-100) and adoptive transfer experiments have demonstrated that T_{reg} can suppress T1D in BBdp rats (38; 101). However, other anti-inflammatory immune cells with tolerogenic properties also contribute

to immune homeostasis, including some innate immune populations such as M2 macrophages (102; 103).

M2 macrophages: Resident macrophages in the gastrointestinal tract are required for maintaining tolerance towards commensal microbes and dietary factors (104). Specifically, M2 macrophages are a subset that play a critical role in maintaining intestinal homeostasis by promoting a tolerogenic immune phenotype (105; 106). The macrophage designations 'M1' and 'M2' are associated with T_H1- and T_H2-biased immune phenotypes characterized by the hallmark cytokines IFN-γ and IL-4, respectively (107). "Classically activated" M1 macrophages are associated with an activated pro-inflammatory immune phenotype, characterized by increased IL-12 production, NO production, as well as high antigen-presenting and co-stimulatory capacity (108). Conversely, "alternatively activated" M2 macrophages are associated with maintenance of an anti-inflammatory state, growth, tissue repair, vascularization, phagocytosis, apoptotic debris removal, and wound healing (108). CD163 is a surface scavenger receptor and marker of mature tissue resident M2 macrophages (109). CD163 is considered a marker of M2c macrophages (110-112), a subset of anti-inflammatory M2 macrophages specifically implicated in regulation of wound healing, tissue repair, and immunomodulation (113). The strongest functional evidence of tolerogenic M2 macrophage capacity to inhibit T1D was reported in a recent study by Parsa *et al*. (102). Adoptive transfer of M2 macrophages into NOD mice inhibited T1D, demonstrating the strong immunomodulatory capacity of these cells (102).

Islet homeostasis in T1D pathogenesis

β-cells represent the primary targets of islet autoimmunity and therefore play a central role in T1D pathogenesis (1). During T1D development, there is a gradual decline in β-cell mass (114; 115); when patients are diagnosed, up to 80% of β-cell mass has been lost (43), mainly by apoptosis (116). Islet infiltration by macrophages and T cells and subsequent immune cell interaction with β-cells, either through direct contact or secretion of pro-inflammatory mediators, contribute to islet destruction (117). IFN-γ promotes β-cell apoptosis, underscoring the importance of T_H1-biased immune responses in T1D (43).

Restoring endogenous insulin secretion and achieving insulin independence is a primary goal of T1D research. As the decrease in β-cell mass is a gradual destructive process, there is likely an opportunity to target residual cells for expansion (118). In NOD mice, feeding a protective diet lacking wheat proteins was associated with increased pancreatic expression of the *Il4* gene, suggesting a benefit of pancreatic T_H2-biased immune responses (70). Diet could play a role in modulating islet homeostasis. In an intervention trial involving islet autoantibody-positive first-degree relatives of patients with T1D, six months of gluten avoidance resulted in a significant increase in the first-phase insulin response, suggesting a beneficial effect of a gluten-free diet on β-cell function (119). The benefit of gluten avoidance from the previous study could be associated with induction of anti-inflammatory immune pathways, suggesting the potential for a window for recovery of β-cell function in patients with signs of islet autoimmunity. Consistent with this concept, enhanced C-peptide concentration and temporary insulin independence was achieved in many newly-diagnosed T1D patients following bone marrow transplantation (120). Thus, dietary or

immune interventions, even post-diagnosis, have the potential to afford at least temporary benefit, by limiting pro-inflammatory immune responses and increasing the function of residual β-cells.

The usually low rates of β-cell proliferation in postnatal rodents can be stimulated to increase when inflammation is present. For example, β-cell proliferation was observed to gradually increase in NOD mice throughout T1D progression (121). In contrast, proliferation was not modified in immunodeficient NOD (NOD/*scid*) mice that lacked islet inflammation (121), suggesting involvement of pro-inflammatory immune factors in stimulating β-cell expansion. The process of islet repair in the face of chronic islet autoimmune attack could serve to amplify β-cell autoimmunity by providing new targets and epitopes for pathogenic effector immune cells (115). Interestingly, Sreenan *et al*. reported enhancement of β-cell proliferation in parallel with decreased β-cell mass preceding T1D (122). Similarly, our group reported enhanced islet expansion processes in the pancreata of young BBdp rats compared with BBc rats that preceded insulitis (123). Thus, excessive islet expansion in young diabetes-prone rodents could be an important target tissue abnormality preceding classic insulitis that promotes T1D development.

Summary

T1D potentially develops by different pathways in a heterogeneous fashion, with progression in any particular individual dependent on a multitude of intersecting genetic factors and environmental exposures (2). However, defective intestinal homeostasis involving a non-exclusive combination of impaired barrier function, regulatory/pro-inflammatory immune

imbalance, and altered microbiota could be a fundamental feature of T1D pathogenesis. In parallel, intrinsic abnormalities in the target pancreas could also contribute to T1D. Thus, gut-originating inflammation could culminate in the activation of an adaptive immune response with specificity for pancreatic β-cells. Targeting of gut immunity and islet homeostasis, for example by dietary modification, could be effective in preventing or treating T1D.

RESEARCH OVERVIEW

Hypotheses

(i) Environmental factors that influence T1D incidence in diabetes-prone rats function in part by modifying small intestinal inflammation; (ii) Target tissue abnormalities precede development of classic insulitis in the pancreata of diabetes-prone rats.

Research Questions

1. *Is environmental alteration of T1D by diet and microbes reflected by modification of the immune infiltrate in the jejunum of BBdp rats?* [*Chapter 3*]

2. *Is dietary alteration of T1D reflected by modification of the immune infiltrate in the jejunum of LEW-DP rats?* [*Chapter 4*]

3. *Can diet-modifiable immune factors be identified in the small intestine of BBdp rats fed diabetes-modifying diets using gene expression screening?* [*Chapter 5*]

4. *Are there early target tissue abnormalities that can be identified in the pancreata of BBdp rats using a prospective partial pancreatectomy strategy?* [*Chapter 6*]

CHAPTER 2. Materials and Methods

Ethics approval

The Animal Care Committees at the University of Ottawa/OHRI and Health Canada approved the studies. Animals were maintained according to the Canadian Council on Animal Care guidelines.

Animal studies - Housing and diets

BBdp rats (SPF condition): BBdp rats were maintained at the OHRI Animal Facility (Loeb building) or at the Animal Resources Division of Health Canada (Ottawa, Canada). Animals were kept in protected laminar flow cages under SPF conditions. 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, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse polio virus, *Encephalitozoon cuniculi*, Hanta virus, CAR bacillus, and rat parvovirus. At 23 d, rats were weaned onto either an HC-based AIN93G diet or cereal-based diets, either an NTP-2000 diet (Ziegler Bros, Gardners, USA) or a Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, Montreal, Canada).

BBdp rats (Germ-free condition): Germ-free BBdp rats were derived from SPF-housed BBdp rats by caesarean section. Term SPF BBdp mothers were anaesthetized and hysterectomies were performed. Germ-free BBdp pups were nursed by germ-free Sprague Dawley foster mothers (IFFA-CREDO, Lyon, France; distributed by Charles River, Montreal, Canada). Sterile germ-free environments were maintained using plastic shoebox cages with isolator

caps inside closed isolator hoods kept under positive pressure and equipped with fans circulating HEPA-filtered air; also, sterile wood bedding and sterile irradiated water were used (Charles River, Wilmington, USA). Animals were fed either an irradiated AIN93G HC diet sterilized with gamma irradiation ranging from 34.5 kGy to 46.6 kGy at MDS Nordion (Montreal, Canada) and stored at 4º C or an irradiated cereal-based C.R. Rodent 18% diet (PMI Nutrition International, Brentwood, USA), which was vacuum packed in 5 pound bags (Zeigler Bros, Gardners, USA); the companies guarantee that the diets were free of bacteria. Virus-free status of germ-free BBdp rats was confirmed by enzyme-linked immunosorbent assay (ELISA) for antibodies against several viruses. Fresh fecal pellets from the isolation chambers were tested for the presence of live bacteria in germ-free BBdp rats. Denaturing gradient gel electrophoresis was performed to confirm sterile status (124; 125). Bacterial DNA was isolated from fecal samples using the QIAamp DNA Stool Mini kit (Qiagen, Mississauga, Canada). Genes encoding bacterial 16S ribosomal RNA were amplified using the universal primers F27 and R1492, as previously described (126). Examination of samples from germ-free BBdp rats did not reveal the presence of 16S rRNA genes, confirming the absence of bacteria.

Lewis rats: Diabetes-prone LEW.1AR1-*iddm* (LEW-DP) and control LEW.1AR1 (LEW-C) rats were obtained from Dr. S. Lenzen at the Institute for Laboratory Animal Science at the Hannover Medical School (Hannover, Germany). Rats were maintained at the Ottawa Hospital Research Institute. At 23 d, rats were weaned onto either a cereal-based diet (Teklad Global 18% Protein rodent diet; Harlan, Montreal, Canada) or an HC-based AIN93G diet.

Monitoring of diabetic status

Rats were weaned at 23 d and provided free access to either cereal-based diets or an HC diet. Beginning at 55 d, rats were tested twice weekly for glucosuria. Rats with glucosuria were fasted overnight, and blood glucose was measured the next morning using a glucometer. T1D was diagnosed when fasting blood glucose was ≥ 11.1 mM; rats were monitored for T1D until at least 134 d. Within 48 hours of T1D diagnosis, or in rats remaining asymptomatic (134-149 d for BBdp; 100 d for LEW-DP), rats were anaesthetized using 2.5% halothane (or 4% isoflurane) in oxygen, weighed, and exsanguinated from the abdominal aorta. Jejunal tissue was cut open longitudinally, rinsed with PBS, rolled up, and fixed in Bouin's fixative (immunohistochemistry) or snap-frozen in liquid nitrogen (gene expression). Following fixation for 24 hours, tissues were transferred to a 75% ethanol solution, dehydrated, and embedded in paraffin for histological studies.

Injections of CAMP/LL-37 in young BBdp rats

BBdp rats were treated with cathelicidin antimicrobial peptide (CAMP)/LL-37 (AnaSpec, Fremont, USA) to study its pleiotropic effects on intestinal microbes, pancreas, and immune parameters. At 23 d, animals were weaned onto a cereal-based diet (Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Montreal, Canada). At 24 d, BBdp and BBc rats were injected (i.p.) daily for one week with either CAMP/LL-37 (1 mg/kg; n=10/group) or saline (n=10/group) (total 40 animals). Fecal samples were obtained following the treatment course, flash frozen in liquid nitrogen, and stored at -80^{o} C. DNA was extracted from the fecal samples and subjected to high-throughput metagenomic analyses (Dr. A. Stintzi, University of Ottawa, Ottawa, Canada). At 30 d, BBdp rats were killed; jejunum, pancreas, PLN, spleen, and liver were harvested.

Partial pancreatectomy (PPx) studies

We performed a unique pancreatic biopsy study using BBdp rats to explore target tissue features present in the pancreata of susceptible animals during the period preceding insulitis. At 23 d, BBdp rats were weaned onto a cereal-based diet (Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Montreal, Canada). Pancreas samples from pre-insulitic BBdp rats were obtained upon 30% partial pancreatectomy (PPx) at 30 days of age. The first cohort of BBdp rats underwent PPx (~30% pancreas) and the excised tissue was placed in Universal Molecular Fixative (UMFIX) for immunohistochemistry experiments. The second cohort underwent PPx and tissue was used for RNA isolation for gene expression studies. Details about the pancreatic RNA isolation protocol appear later in this *Chapter*. Sham operations were also performed but the pancreas was left intact. In total, n=26 animals had PPx surgery and n=23 had a sham surgery. *Note: I isolated the RNA and performed a subset of sham operations while Dr. G.-S. Wang performed all PPx surgeries and most sham surgeries.* At 150 d (endpoint), all pancreatectomized rats had either turned diabetic or remained asymptomatic and corresponding 30 d samples (tissue or RNA) were retrospectively classified as either “pre-diabetic” or “resistant”.

Insulin concentration in the serum

Serum insulin was evaluated by ELISA using rat insulin 96-well microplate ELISA kit (catalog number 10-1250-01; Mercodia, Uppsala, Sweden). The kit is based on a direct sandwich technique, which uses two monoclonal antibodies recognizing distinct rat insulin epitopes. During the reaction, insulin proteins bind to separate plate-bound antibodies as well as peroxidase-conjugated antibodies, both directed against insulin. Detection of bound conjugated antibody was achieved by adding tetramethylbenzidine as the substrate. A

standard calibration curve was generated using kit components for determination of insulin concentration in the BBdp rat serum samples. Optical density readings were obtained at 450 nm using a Multiskan Ascent v1.24 (ThermoFisher Scientific, Inc., Waltham, USA).

Immunohistochemistry of intestinal immune markers using DAB

Jejunum sections were stained using hematoxylin and eosin (H&E) (Pathology Department, University of Ottawa, Ottawa, Canada) and were used for intestinal histological scoring. Eosinophils were labeled using Chromotrope 2R (127). For immunohistochemistry of immune markers, sections were deparaffinized using CitriSolv solution (ThermoFisher Scientific, Inc., Waltham, USA) and rehydrated in ethanol solutions (100%, 95%, 70% ethanol) and water. Antigen retrieval was performed using heat, pressure, and chemical treatment (see **Table 2.1**) in a decloaking chamber (BioCare Medical, Concord, USA). Endogenous tissue peroxidases were inhibited using a solution of 3% H_2O_2 prepared in methanol. Next, sections were incubated with either Universal Blocking Solution (DAKO Canada, Inc., Burlington, Canada) or 5% bovine serum albumin (BSA) buffer (5 g BSA per 100 mL 0.01M PBS; pH=7.6) for 30 minutes for blocking of non-specific binding sites.

Primary antibodies for immunohistochemistry using DAB

The following primary antibodies were used for labeling (see **Table 2.1** for additional technical details). Goat anti-CCL11 (eotaxin-1) (R&D Systems, Minneapolis, USA) polyclonal antibody was applied at a dilution of 1:150. Rabbit anti-CD3 (Abcam, Toronto, Canada) polyclonal antibody at 1:400. Mouse anti-CD8α (BD Biosciences, Mississauga, Canada) polyclonal antibody at 1:10. Rat anti-Foxp3 (eBioscience, San Diego, USA) monoclonal antibody at 1:100. Goat anti-CD163 (Santa Cruz Biotechnology, Santa Cruz,

USA) polyclonal antibody at 1:100. Mouse anti-CD68 (AbD Serotec, Raleigh, USA) monoclonal antibody at 1:50. Goat anti-CD83 (Santa Cruz Biotechnology, Santa Cruz, USA) polyclonal antibody at 1:200. Rabbit anti-cathelicidin (Abcam, Toronto, Canada) polyclonal antibody at 1:3000. Mouse anti-$p16^{INK4a}$ (Abcam, Toronto, Canada) monoclonal antibody at 1:4000. Mouse anti-PCNA (DAKO Canada, Inc., Burlington, Canada) monoclonal antibody at 1:200. Primary antibody incubations were performed at room temperature. Corresponding biotinylated secondary antibodies (DAKO Canada, Inc., Burlington, Canada) were applied for 30 minutes at room temperature followed by incubation with a solution of streptavidin-conjugated horseradish peroxidase (DAKO Canada, Inc., Burlington, Canada) for 30 minutes at room temperature. For reaction visualization, 0.06% 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Canada, Oakville, Canada) and 0.03% H_2O_2 were applied as substrates for colour development. Counterstaining of nuclei was performed using hematoxylin (Electron Microscopy Sciences, Hattfield, USA).

Table 2.1. Primary antibodies and retrieval conditions for staining using DAB protocol

Primary antibody	Company and catalog number	Antibody isotype	Antigen retrieval & antibody dilution
CD3	Abcam; Ab5690	Rabbit polyclonal IgG	Boiling 0.1M citric acid pH=6.0 (30 min); 1:400
CD8α	BD Pharmingen; BD550298	Mouse monoclonal IgG	Boiling 0.1M citric acid pH=6.0 (10 min); 1:10
Foxp3 (FJK-16s)	eBioscience; 14-5773-82	Rat monoclonal IgG	Boiling 1 mM EDTA pH=8.0 (30 min); 1:100
CD163 (G-17)	Santa Cruz Biotechnology; sc-18795	Goat polyclonal IgG	Boiling 0.1M citric acid pH=6.0 (10 min); 1:100
CD68	AbD Serotec; MCA341GA	Mouse monoclonal IgG	*Jejunum*: Boiling 0.1M citric acid pH=6.0 (10 min); 1:50; *Pancreas*: Boiling 0.1M citric acid pH=6.0 (4 min); 1:400
Cathelicidin/CAMP	Abcam; ab74946	Rabbit polyclonal IgG	Boiling 0.1 M citric acid pH=6.0 (10 min); 1:3000
$p16^{INK4a}$	Abcam; ab54210	Mouse monoclonal IgG	Boiling 1 mM EDTA pH=9.0 (4 min); 1:4000
PCNA (PC10)	DAKO; M0879	Mouse monoclonal IgG	Boiling 0.1 M citric acid pH=6.0 (4 min); 1:200

Microscopy and imaging of small intestinal tissue

Light microscopy was performed using an Axioplan2 light microscope (Zeiss Canada, Mississauga, Canada) and image capturing for morphometric analyses was performed using a CCD color camera (QImaging, Surrey, Canada). Northern Eclipse morphometry software (Empix Imaging, Mississauga, Canada) was used for measurements of tissue area and quantification of cells labeled by immunohistochemistry. Using coded slides, stained cells in the jejunum were quantified on a minimum of 10 fields per tissue section from images captured at 200x magnification. The ScanScope CS eSlide capture device and ImageScope software (Aperio, Vista, USA) were also used for some analyses.

Evaluation of small intestinal histological enteropathy

Histology in the jejunum was evaluated using an adaptation of the Marsh classification scale (128) used for clinical evaluation for small intestinal histopathology associated with celiac disease. The five following parameters were scored: **1.** *Goblet cell homeostasis* (normal: 0, abnormal: 1); **2.** *Villus morphology* (normal: 0, decreased height: 1, flattened: 2); **3.** *Crypt morphology* (normal: 0, increased height: 1); **4.** *Immune cell infiltration* (normal: 0, infiltration in lamina propria: 1, infiltration in lamina propria + submucosa: 2, infiltration in lamina propria, submucosa, and muscularis propria: 3); **5.** *Muscularis thickness* (normal: 0, mild thickening: 1, severe thickening: 2). Total histological scores were determined, which ranged from 0 (normal histology) to 9 (severe enteropathy).

Immune cell morphometry

For quantification of immune cells in the gut epithelium of the jejunum, average epithelial cell width was determined in adult rats; 800 nuclei were counted and villus length measured in 24 villi for estimation of the average width of a single rat epithelial cell, which was 7.5 μm. The number of epithelial cells in the villus was determined by dividing the total length of the analyzed villus segment by the width of a single epithelial cell (7.5 μm). For determination of $CD3^+$ and $CD8\alpha^+$ IEL densities as well as epithelium-associated $CD163^+$ cells, only straight elongated villus segments were analysed; crypts were excluded from all measurements. $CD3^+$ IEL, $CD8\alpha^+$ IEL, and epithelium-associated $CD163^+$ cells were expressed as the number of cells per 100 villus epithelial cells, as performed for clinical evaluation of celiac disease (128). For measurement of cells in the lamina propria, $CD3^+$ cells (LPL), $CD8\alpha^+$ cells (LPL), $Foxp3^+$ cells, $CD163^+$ cells, and $CD68^+$ cells were counted

manually and expressed as the cell number per mm^2 jejunum area, which excluded the submucosa and muscularis propria; $Foxp3^+$ cells were also expressed as a percentage of $CD3^+$ LPL.

Immunofluorescence and confocal microscopy

Immunofluorescence was performed using a Zeiss LSM 510 Meta laser scanning confocal microscope (Zeiss Canada, Mississauga, Canada). Guinea-pig anti-insulin (A0564; DAKO Canada, Inc., Burlington, Canada) polyclonal antibody was used. For information about the other antibodies used for immunofluorescence, see **Table 2.1**. Secondary antibodies conjugated to the following fluorophores were used: Alexa488, FITC, Cy3, and Cy5 (Jackson ImmunoResearch, West Grove, USA). For some stainings, biotinylated secondary antibodies (DAKO Canada, Inc., Burlington, Canada) were applied followed by streptavidin-conjugated Cy3 (Jackson ImmunoResearch, West Grove, USA). Hoechst (Sigma-Aldrich Canada, Oakville, Canada) was used for nuclear staining. See **Table 2.2** for detailed description of antibodies and retrieval conditions used for immunofluorescence stainings. LSM files were processed using ZEN 2009 software (Zeiss Canada, Mississauga, Canada).

Table 2.2. Primary antibodies and retrieval conditions for immunofluorescence

Primary antibodies and dilution	Secondary antibodies/fluorophores and dilution	Antigen retrieval
CAMP (1:200); CD163 (ED2)(1:10); CD14 (1:50) (*Jejunum*)	CAMP: Donkey anti-rabbit-Cy3 (1:300); CD14: Donkey anti-goat Alexa488 (1:200); CD163 (ED2): Rabbit anti-mouse-Cy5 (1:200)	Enzyme treatment (0.05% trypsin pH=7.6, 10 min)
CAMP (1:300); CD68 (1:10) (*Jejunum, PLN, and pancreas*)	CAMP: Goat anti-rabbit (DAKO/EO432; 1:300) + streptavidin-Cy3 (1:300) CD68: Donkey anti-mouse Alexa488 (1:200)	Boiling 0.1 M citric acid pH=6.0 (10 min)
CAMP (1:200); CD14 (1:50) (*PLN*)	CAMP: Donkey anti-rabbit-Cy3 (1:300); CD14: Donkey anti-goat Alexa488 (1:200)	Boiling 0.1 M citric acid pH=6.0 (10 min)
CAMP (1:300); CD163 (G-17)(1:30) (*PLN*)	CAMP: Donkey anti-rabbit-Alexa488 (1:200); CD163 (G-17): Rabbit anti-goat (DAKO/EO466; 1:600) + streptavidin-Cy3 (1:300)	Boiling 0.1 M citric acid pH=6.0 (10 min)
CAMP (1:200); INSULIN (1:50)	CAMP: Donkey anti-rabbit-Cy3 (1:300); INSULIN: Donkey anti-guinea pig-FITC (1:200)	Boiling 0.1 M citric acid pH=6.0 (10 min)
CAMP (1:200); CD68 (1:10); INSULIN (1:50)	CAMP: Donkey anti-rabbit-Cy3 (1:300); CD68: Rabbit anti-mouse-Cy5 (1:200); INSULIN: Donkey anti-guinea pig-FITC (1:200)	Boiling 0.1 M citric acid pH=6.0 (10 min)
$p16^{INK4a}$ (1:32,000); INSULIN (1:50)	$p16^{INK4a}$: Rabbit anti-mouse (DAKO/EO354; 1:300) + streptavidin-Cy3 (1:1200); INSULIN: Donkey anti-guinea pig-FITC (1:200)	Boiling 1 mM EDTA pH=9.0 (4 min)

RNA isolation

For standard gene expression experiments (TaqMan Gene Expression Assays): Total RNA was isolated from frozen jejunum samples using TRIzol reagent (Invitrogen, Life Technologies, Burlington, Canada). Briefly, TRIzol reagent was added to frozen jejunum (1 mL per 50-100 mg sample); tissue represented ~10% of the TRIzol reaction mixture volume.

Homogenization: Tissue was disrupted at room temperature for 15 seconds by mechanical homogenization using an electric handheld homogenizer. *Phase separation*: Homogenized samples were incubated at room temperature for 5 minutes to enable full dissociation between nucleic acids and proteins. Chloroform was added (200 µL per 1 mL initial TRIzol) and tubes were briefly vortexed and shaken vigorously for 15 seconds; samples were incubated for 3 minutes at room temperature. Next, samples were centrifuged at 12,000 x *g* at 4^{o} C for 15 minutes using an Eppendorf 5417R centrifuge (Eppendorf Canada, Mississauga, Canada). The upper colorless aqueous phase containing the RNA was carefully removed (~1 mL) and transferred to a new tube; the lower red phenol-chloroform phase and white interphase layer were discarded. *RNA precipitation*: Isopropanol (500 µl per 1 mL initial TRIzol) was added to the aqueous phase and samples were incubated for 10 minutes at room temperature. Samples were centrifuged at 12,000 x *g* at 4^{o} C for 10 minutes. *RNA washing*: Supernatant was removed from the RNA pellet, which was washed using 1 mL ethanol (75% in DEPC-H_2O), briefly vortexed, and centrifuged at 7,500 x *g* at 4^{o} C for 5 minutes; the supernatant was discarded. The RNA pellet was air dried for 5 minutes and resuspended in 200 µL nuclease-free H_2O. RNA concentration was determined using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech/GE Healthcare, Uppsala, Sweden). Samples were stored at -80^{o} C.

Reverse transcription

For synthesis of complementary DNA (cDNA), isolated RNA (2 µg) was reverse transcribed in a final reaction mixture volume of 40 µL containing Invitrogen reagents (Invitrogen, Life Technologies, Burlington, Canada). The mixture consisted of 1X First Strand Buffer, 0.01 M dithiothreitol (DTT), 0.5 mM dNTP, 25 µg/mL Oligo$(dT)_{25}$ as template primer, 2 U/µL

RNaseOUT recombinant ribonuclease inhibitor, and 10 U/μL (M-MLV) reverse transcriptase. The reverse transcription reaction was carried out at 42° C for 90 minutes, 94° C for 5 minutes, and 4° C for 10 minutes. Thermocycling was done using an Eppendorf Mastercycler (Eppendorf Canada, Mississauga, Canada). Samples were stored at -20 ° C.

Real-time polymerase chain reaction (PCR) using TaqMan Gene Expression Assays

Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Life Technologies, Burlington, Canada) and experiments were carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies, Burlington, Canada). The thermal profile was as follows: *Stage 1*. 50° C for 2 minutes (1 cycle); *Stage 2*. 95° C for 10 minutes (1 cycle); *Stage 3*. 95° C for 15 seconds followed by 60° C for 1 minute (40 cycles). The reaction mixture contained 1X TaqMan Universal PCR Master Mix, 1X TaqMan Gene Expression Assay Mix consisting of primers and probes for the gene-of-interest and endogenous housekeeping control gene β-actin (*Actb*) (see **Table 2.3**); 6075 ng of template cDNA was used in a final volume of 21 μL per replicate. The reaction mixture contained fluorogenic oligonucleotide probes containing FAM- and VIC-conjugated reporter dyes to enable simultaneous detection of genes-of-interest and the endogenous housekeeping control gene, respectively. For reagent testing, standard curves were performed for the gene-of-interest and housekeeping control gene in singleplex (one assay/reaction; one primer pair and probe set) and multiplex (two assays/reaction; two primer pairs and probe sets) to verify that reaction performance and amplification efficiencies were equivalent. For sample analysis, multiplex PCR reactions were performed in triplicate on a 96-well plate containing triplicates of cDNA calibrator sample and control containing no template.

The comparative C_T method ($\Delta\Delta C_T$) was used for quantitative analysis. The C_T value was defined as the cycle number at which the detected fluorescence meets the threshold value set above background fluorescence. ΔC_T corresponded to the difference between the raw C_T values of the target gene and endogenous control. The ΔC_T value was obtained by subtracting the Ct value of β-actin from the gene-of-interest and the triplicate ΔC_T values were averaged for each unknown sample; the ΔC_T value was obtained for unknown samples and the calibration sample, which was jejunum tissue from an age-matched cereal-fed Wistar Furth (WF) rat. The ΔC_T value of the WF calibration sample was subtracted from each unknown BBdp ΔC_T value to obtain the $\Delta\Delta C_T$ value; the $\Delta\Delta C_T$ value corresponded to the difference between the ΔC_T value between the BBdp sample and WF calibration sample. Expression of the gene-of-interest was normalized to expression of the *Actb* housekeeping gene; results were expressed as relative fold-change amounts ($2^{-\Delta\Delta Ct}$) standardized to the calibrator WF rat sample. For graphical presentation in *Chapter 3* figures, the average fold change values from the Germ-free/HC group were normalized to 1 and averages from other groups were adjusted by the same factor.

Table 2.3. Gene expression analyses in jejunum of BBdp rats

Gene symbol	Corresponding protein	NCBI reference sequence	Applied Biosystems TaqMan Gene Expression Assay catalog number
Foxp3	Forkhead box P3 (Foxp3)	NM_001108250.1	Rn01525092_m1
Ctla4	Cytotoxic T lymphocyte-associated protein 4 (CTLA-4)	NM_031674.1	Rn00581545_m1
Cd163	CD163	NM_001107887.1	Rn01492519_m1
Itgae	Integrin, alpha E (CD103)	NM_031768.1	Rn04224678_u1
Il10	Interleukin 10 (IL-10)	NM_012854.2	Rn00563409_m1
Tgfb1	Transforming growth factor beta 1 (TGF-β1)	NM_021578.2	Rn00572010_m1
Il4	Interleukin 4 (IL-4)	NM_201270.1	Rn01456866_m1
Ifng	Interferon gamma (IFN-γ)	NM_138880.2	Rn00594078_m1
Il15	Interleukin 15 (IL-15)	NM_013129.2	Rn00689964_m1
Il17a	Interleukin 17A (IL-17A)	NM_001106897.1	Rn01757168_m1
Il23	Interleukin 23, subunit p19 (IL-23)	NM_130410.2	Rn00590334_g1
Camp	Cathelicidin	NM_001100724.1	Rn01446022_g1
Actb	Actin, beta (β-actin)	NM_031144.2	4352340E

RNA isolation and cDNA synthesis for intestinal studies using PCR arrays

Total RNA was isolated from whole jejunum using the Nucleospin RNA II RNA Isolation Kit (Macherey-Nagel, Duren, Germany). Following extraction, RNA sample quality and quantity were evaluated using a 6000 Nano LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Canada). For PCR array studies, the RT^2 First Strand Kit (Qiagen/SABiosciences, Mississauga, Canada) was used to convert 1 µg of RNA into cDNA. The cDNA was combined with RT^2 qPCR Master Mix and aliquoted onto 96-well PCR array plates (Qiagen/SABiosciences, Mississauga, Canada).

Pancreatic RNA isolation for PPx-based prospective gene expression screening

The original plan for prospective pancreas gene expression studies (see *Chapter 6*) was to use RNA from islets isolated by laser capture microdissection. However, testing of RNA

quality from pancreas fixed using the intended fixative (Universal Molecular Fixative; UMFIX, Sakura Finetek, Torrance, USA) revealed a high degree of degradation. The UMFIX-associated degradation was pancreas-specific, as other tissues such as liver or gut (not shown) did not display extensive degradation. It was concluded that pancreatic RNA could not be adequately preserved upon fixation using UMFIX or other fixatives including Bouin's, formalin, and RCL-2. Thus, the decision was made to perform partial pancreatectomy with another cohort of BBdp rats and to isolate RNA using a different strategy. As an alternative, the entire pancreas biopsy was homogenized at the time of surgery followed by immediate flash freezing in liquid nitrogen; additional steps in the RNA isolation protocol were subsequently performed. This approach provided good quality RNA and enabled analysis of whole pancreas gene expression (**Appendix A.1**). Thus, the following steps were performed to obtain pancreatic RNA upon PPx: Sterile 15 mL tubes were labeled and filled with appropriate volumes of RA1 lysis buffer (Nucleospin RNA L kit; Macherey-Nagel, Duren, Germany) and β-mercaptoethanol; the tubes were then placed into a bucket of crushed ice for cooling prior to receiving the pancreatectomized tissue. Upon surgical removal of the pancreatectomized sample, the tissue was immediately placed into the pre-cooled 15 mL tube containing a mixture of reducing agent β-mercaptoethanol and RA1 lysis buffer. Without delay, the sample-solution mixture was mechanically homogenized for 12-15 seconds using a Polytron PT-2100 homogenizer (Kinematica, Bohemia, USA). After homogenization, the tube was sealed and the freshly homogenized lysate mixture was flash-frozen and stored in liquid nitrogen. All 14 samples were sequentially procured and processed in this fashion. The tubes containing the homogenized lysates remained submerged in liquid nitrogen until being thawed for continuation of the RNA isolation process as outlined by manufacturer's instructions (Macherey-Nagel, Duren,

Germany). The centrifuge temperature was maintained at 4°C during the RNA isolation process. Upon elution in nuclease-free H_2O, RNA was aliquoted for quantification and quality assessment with additional amounts aliquoted for downstream applications; samples were stored at -80°C. RNA samples were evaluated using a 6000 Nano LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Canada); evaluation performed at OHRI Stemcore laboratories (OHRI, Ottawa, Canada). At 150 d (endpoint), all pancreatectomized rats had either turned diabetic or remained asymptomatic and were accordingly categorized retrospectively as either "pre-diabetic" or "resistant".

PCR Arrays in jejunum and pancreas

Real-time PCR reactions were performed using a cocktail containing RT^2 Real-Time SYBR Green/ROX qPCR Master Mix (Qiagen/SABiosciences, Mississauga, Canada), nuclease-free water, and cDNA. In total, eight RT^2 Profiler PCR Arrays (PARN-052A, Qiagen/SABiosciences, Mississauga, Canada) for "Innate and Adaptive Immune Responses" (*Chapter 5*) were used to evaluate gene expression in intestinal tissue from four biological replicates per dietary group (4 HC-fed and 4 cereal-fed rats). These PCR arrays contained primers for 84 immune genes, including cytokines, chemokines, T_H-associated factors, pattern recognition receptors, and host defense factors. An ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies, Burlington, Canada) was used for the analyses. The thermal profile was as follows: *Stage 1*. 95^o C for 10 minutes (1 cycle); *Stage 2*. 95^o C for 15 seconds followed by 60^o C for 1 minute (40 cycles); *Stage 3 (dissociation)*. 95^o C for 15 seconds followed by 60^o C for 20 seconds and 95^o C for 15 seconds (1 cycle). Baseline and threshold values were set manually according to the

manufacturer's instructions. Genes with Ct values over 35 were considered undetectable. Ct values for genes-of-interest were normalized with the average Ct values of five housekeeping genes: Ribosomal protein, large, P1 (*Rplp1*), hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), ribosomal protein L13a (*Rpl13a*), lactate dehydrogenase A (*Ldha*), and β-actin (*Actb*). Results are shown as fold-change ($2^{-\Delta\Delta Ct}$), comparing the effects of low-antigen HC feeding with cereal feeding. In *Chapter 6*, the RT^2 Profiler PCR arrays used were entitled "Rat Inflammatory Response and Autoimmunity" (PARN-077A, Qiagen/SABiosciences, Mississauga, Canada), for comparing the effects of pre-diabetic BBdp pancreata with diabetes-resistant BBdp pancreata. RT^2 Profiler PCR Array Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) was used to analyze the data and generate volcano plots and scatter plots.

Microarrays

Microarrays (GeneChip Rat Gene 1.0 ST Arrays, Affymetrix, Santa Clara, USA) were used to compare gene expression in prospective 30 d pre-diabetic and resistant BBdp rat pancreata. These microarrays provide extensive coverage of the rat transcriptome (median 22 probes/gene), with over 24,000 genes (Affymetrix). For the experiment, two pooled RNA preparations were used (n=7/group). Two 5 µl preparations of pooled RNA (100 ng/µL) were submitted for microarray analysis at OHRI Stemcore laboratories (OHRI, Ottawa, Canada). A. Noel (Scott lab) generated the gene list by processing the CEL files using AltAnalyze software (129) (http://www.altanalyze.org; Gladstone Institutes, UCSF).

Cell culture

The rat insulinoma INS-1E cell line (130; 131) was used for the study of β-cells *in vitro*. Cells were grown at 5% CO_2/95% air at 37^o C. Cells were passaged weekly following trypsin treatment (0.5% Trypsin-EDTA, Gibco, Life Technologies, Burlington, Canada). Experiments were performed between passages 52 and 64.

Culture media for rat insulinoma INS-1E cells:

- RPMI-1640 medium (Invitrogen, Life Technologies, Burlington, Canada)
- 5% Fetal Bovine Serum (Sigma-Aldrich Canada, Oakville, Canada)
- 100 IU/mL penicillin, 100 ug/mL streptomycin (Pen Strep, Gibco, Life Technologies, Burlington, Canada)
- 1 mM sodium pyruvate (Gibco, Life Technologies, Burlington, Canada)
- 50 μM β-mercaptoethanol

Statistics

For survival analysis, the log rank test was performed. Comparisons among groups were made using Student's *t*-test or one-way ANOVA with least significant difference (LSD) post-hoc test for determination of significance of differences among multiple means. Data are presented as means ± standard deviation (SD). For correlation analyses, R coefficients were obtained using Pearson's correlation; *p*-values obtained by linear regression. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using Statistica v6.0 (Statsoft, Inc., Tulsa, USA) and GraphPad Prism v4.02 (GraphPad Software, Inc., La Jolla, USA).

CHAPTER 3. Gut immune infiltrate parallels environmental modification of autoimmune diabetes

ABSTRACT

Background: Microbes and diet were separated to study the effect of these ubiquitous influences on gut inflammation and T1D development.

Methods: BBdp rats were housed under specific pathogen-free (SPF) or germ-free conditions and weaned onto cereal-based diets or a hydrolyzed casein (HC) diet. Immune cell distribution and immune-associated gene expression were analyzed in jejunum by immunohistochemistry, morphometry, and RT-qPCR.

Results: Cereal-fed rats displayed increased T1D incidence compared with HC-fed counterparts under both SPF conditions (65% vs. 29%) and germ-free conditions (53% vs. 7%). Microbes also promoted T1D, but only in the absence of cereal antigens (29% vs. 7%). In the jejunum of SPF-housed rats, cereal feeding increased $CD3^+$ and $CD8\alpha^+$ T cell infiltration. HC diet-protected rats had increased anti-inflammatory $CD163^+$ M2 macrophages compared with cereal-fed rats. Cereal feeding increased the *Ifng*/*Il4* cytokine mRNA ratio, reflective of enhanced T_H1 transcriptional bias in association with decreased *Cd163* gene expression. When microbes were the main antigens and promoted T1D, T cell infiltration was increased whereas the proportion of $Foxp3^+$ T_{reg} was decreased; *Ifng* transcription was also upregulated.

Summary: The phenotype of the immune infiltrate in the BBdp jejunum reflected environment-associated T1D. Cereal promoted T1D under standard and sterile conditions in conjunction with pro-inflammatory immune activation in parallel with deficits in anti-inflammatory immune cells.

INTRODUCTION

A spectrum of environmental determinants, such as wheat proteins, bacteria, and viruses has been implicated in T1D pathogenesis (3; 5; 132). Cereal-fed rodents develop increased T1D compared with animals fed a low-antigen HC diet or gluten-free formula (60; 133; 134). The mechanism by which diet modulates T1D in diabetes-prone animals is not completely understood but could involve the gastrointestinal tract, a major environment-exposed organ critical for immune homeostasis (135). Accumulating evidence suggests the gut is structurally and immunologically abnormal in a subset of individuals susceptible to T1D (2; 49; 51; 58; 60; 136-138). Our group previously reported that the MLN of BBdp rats were characterized by increased expression of the T_H1 cytokine, IFN-γ (36). A picture is emerging of an interplay between defective intestinal barrier function, environmental exposures, and altered gut immune homeostasis forming the basis of T1D pathogenesis (9-11; 139). The small intestinal immune compartment could represent a primary site where chronic inflammation originates, leading to activation of β-cell autoimmunity. Using NOD mice, Turley *et al*. demonstrated that oral antigen feeding or small intestinal damage resulted in immune cell activation in PLN and insulitis, respectively (61), reinforcing the strong functional association between small intestinal and pancreatic inflammation. Consistent with this concept, a constitutive celiac-like enteropathy is a common feature of BBdp rats and precedes T1D (44); a similar intestinal phenotype has also been described in NOD mice (60). The enteropathy in BBdp rats is partly manifested by increased small intestinal permeability, which precedes T1D (51; 58). Because stimulation of tight junction closure in the small intestine of BBdp rats prevented T1D (58), it is clear that the gut plays a major role in pathogenesis. In patients, signs of enteropathy and increased intestinal permeability have been observed prior to clinical onset of T1D (50) and ultra-structural mucosal abnormalities

have also been reported (140). The small intestinal immune microenvironment, comprising the epithelium and lamina propria, likely represents a major site where environment influences the immune system. In susceptible subjects, chronic inflammation in the small intestine could activate immune cells capable of targeting pancreatic β-cells (2). In support of this view, gut-originating T cells bearing the gut-homing integrin $\alpha_4\beta_7$ were found to infiltrate the pancreata of young NOD mice (64; 65). Exposure to cereal antigens in the context of intestinal immune impairment on a diabetes-prone genetic background could stimulate gut immune activation and promote T1D (81). A previous study by our group found that a subset of patients with T1D displayed enhanced peripheral T cell proliferation and a mixed pro-inflammatory cytokine secretion pattern upon *in vitro* challenge with wheat peptides (82). A major obstacle in understanding the role of diet and gut inflammation in T1D pathogenesis is that the intestinal immune response elicited by dietary antigens occurs in parallel with immune defenses required for homeostasis with commensal microbes and prevention of infection by pathogens. In the present study, the effects of diet and microbes were parsed to analyze their influence on T1D development and gut immune infiltration in BBdp rats. By evaluating these two major environmental factors and examining immune aspects of the jejunal interface *in situ*, the present study afforded new perspectives on T1D and its association with small intestinal inflammation. Several of the results in this *Chapter* were reported in Patrick *et al*., *Diabetes* 2013 (103).

RESEARCH OVERVIEW

Rationale

Mild enteropathy is a constitutive feature of BBdp rats compared with BBc rats. Diet is a major environmental modifier of T1D in BBdp rats. Microbes are major environmental factors also present in the gut along with dietary antigens. Diet and microbes have not been separated to determine their influence on intestinal immune homeostasis and T1D.

Hypotheses

(i) Diet and microbes are ubiquitous environmental factors that both influence spontaneous T1D development in BBdp rats; *(ii) modification of the gut immune infiltrate parallels environmental alteration of T1D, with disease promotion associated with a pro-inflammatory phenotype in the context of impaired immune regulation*.

Research Questions

1. *Is the promotion of T1D by feeding of cereal-based diets associated with a pro-inflammatory gut immune phenotype in SPF and germ-free BBdp rats compared with HC feeding?*

2. *Is microbial exposure required for T1D development and if so, is microbe-associated modification of T1D reflected by alteration of the gut immune infiltrate in BBdp rats?*

3. *Are deficits in small intestinal anti-inflammatory immune cells present in BBdp rats exposed to diabetes-promoting environmental factors?*

RESULTS

Effect of diet and microbes on T1D incidence in BBdp rats

At 23 d, BBdp rats that were either SPF-housed or germ-free were fed either standard cereal diets or a low-antigen HC diet and T1D incidence was determined. Consistent with previous findings from our group (132; 133), BBdp rats housed under SPF conditions had a significantly lower T1D incidence when fed an HC diet (29%) compared with a cereal diet (65%) (**Fig. 3.1A**). In the absence of microbes, dietary modification of T1D incidence was maintained: Germ-free BBdp rats fed an HC diet had a significantly lower T1D incidence (7%) compared with cereal-fed animals (53%). Microbial exposure also had diabetes-promoting capacity in BBdp rats. Among rats fed an HC diet, those exposed to microbes had significantly increased T1D incidence (29%) compared with germ-free rats (7%). Overall, environmental antigenic load in the gut lumen had a major effect on T1D development; high-antigen SPF/Cereal rats had the highest incidence whereas low-antigen Germ-free/HC rats were nearly all protected. Thus, diet and microbes both influenced T1D with cereal antigens providing the strongest diabetes-promoting stimulus.

Effect of diet and microbes on serum insulin

The influence of diet and microbes on fasting circulating insulin concentration in BBdp rats was evaluated in 130 d animals from **Fig. 3.1A** that remained asymptomatic. The highest fasting serum insulin concentration was observed in Germ-free/HC rats whereas the smallest concentration was in SPF/Cereal rats (**Fig. 3.1B**), groups that respectively represented low and high extremes of T1D incidence. A trend of increased serum insulin was observed in HC-fed rats compared with cereal-fed but statistical significance was not reached.

Effect of diet and microbes on jejunum histology

Histological celiac-like enteropathy was previously described in BBdp rats compared with BBc rats (44). Therefore, gut histology was analyzed in the jejunum of BBdp rats to determine whether diet or microbes altered enteropathy. Histological scores were obtained using a modified Marsh classification scale, which is used for clinical evaluation of celiac histopathology (128). No significant differences in histological score were observed (**Fig. 3.1C**). There were sporadic regions with pronounced inflammatory foci only observed in germ-free rats. These inflamed areas were localized to jejunal crypts, submucosal regions, and inner muscularis propria; the inflammation consisted of mixed immune infiltrates featuring enrichment of eosinophils, macrophages and DC (**Fig. 3.1D**). In healthy individuals, the small intestinal mucosa, comprising the epithelium and lamina propria, is populated in part by T cells and macrophages (128). In the following experiments, these populations were quantified by detailed morphometry to evaluate the effect of environmental alteration on cell distribution in the jejunum.

Note: Unless specified, the following experiments (Figs. 3.2-3.8) were performed using animals from Fig. 3.1A that remained asymptomatic until 130 d.

Effect of diet and microbes on $CD3^+$ T cell distribution

Total T cells were evaluated on the basis of $CD3^+$ expression and quantified according to localization in either epithelium (intra-epithelial lymphocytes; IEL) or lamina propria (lamina propria lymphocytes; LPL) (**Fig. 3.2A**). In the jejunum, LPL are typically described as being $CD4^+$, making them distinct from IEL, which are distinguished by enrichment of CD8 and the integrin $\alpha_E\beta_7$ (CD103) (128; 141; 142).

FIGURE 3.1

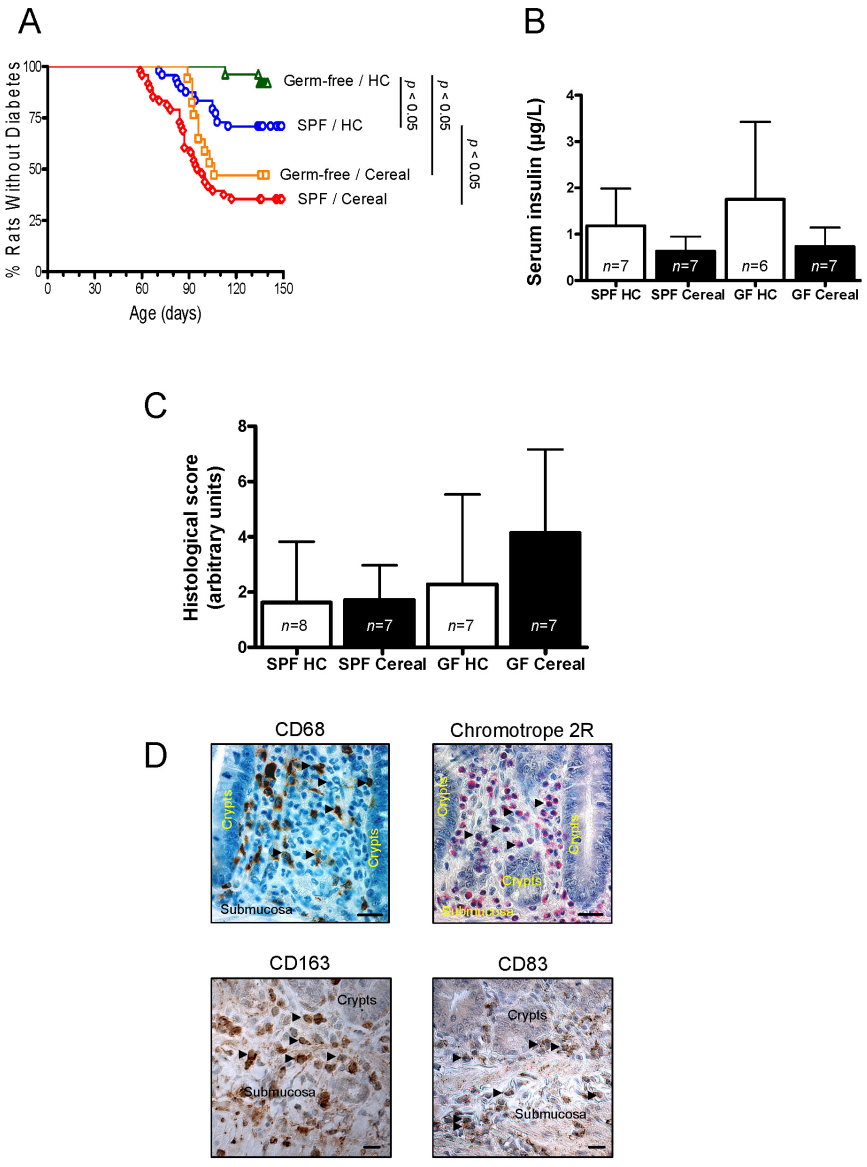

A
% Rats Without Diabetes
Age (days)
Germ-free / HC
SPF / HC
Germ-free / Cereal
SPF / Cereal
p < 0.05
p < 0.05
p < 0.05
B
Serum insulin (µg/L)
n=7
n=7
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
C
Histological score (arbitrary units)
n=8
n=7
n=7
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
D
CD68
Chromotrope 2R
CD163
CD83
Crypts
Submucosa

Figure 3.1. Effect of diet and microbes on T1D and small intestinal histology in BBdp rats

Animals were housed under standard SPF or germ-free conditions, weaned onto standard cereal-based diets or a low-antigen HC diet, and T1D incidence was determined. SPF/Cereal rats (*n*=48), SPF/HC rats (*n*=48), Germ-free/Cereal (*n*=17), Germ-free/HC (*n*=27). (**A**) Kaplan-Meier survival graph indicating the percentage of BBdp rats that remained free of T1D until ~150 d. *P*-values determined using the log-rank test. (**B**) Concentration of fasting serum insulin in 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). *n*=6-7 rats/group. (**C**) Small intestinal histological features were evaluated in the jejunum. Histological scores of BBdp rats exposed to distinct environmental conditions. Histological scores were determined on the basis of villus length, crypt length, immune cell infiltration, and goblet cell number using a modified Marsh scale (see *Chapter 2 - Materials and Methods*). *n*=7-8 rats/group. Data represent mean ± SD. (**D**) Sporadic immune infiltration of crypts and submucosal regions observed in germ-free animals. Mixed immune infiltrates were observed, which were enriched in macrophages (left panels), Chromotrope $2R^{+}$ cells (eosinophils) (upper right panel), and DC (lower right panel). *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

The numbers of both $CD3^+$ IEL and $CD3^+$ LPL were largest in high-antigen SPF/Cereal rats and smallest in low-antigen Germ-free/HC rats (**Figs. 3.2B-C**), conditions that reflected high and low extremes of T1D incidence, respectively (**Fig. 3.1A**). Under SPF conditions, the number of $CD3^+$ IEL was significantly larger in cereal-fed rats compared with HC-fed rats (**Fig. 3.2B**). Consistent with this finding, the number of $CD3^+$ LPL was also significantly increased in cereal-fed compared with HC-fed rats under SPF conditions (**Fig. 3.2C**). Microbial status also influenced $CD3^+$ T cell distribution. Rats exposed to microbes had significantly increased numbers of $CD3^+$ IEL and $CD3^+$ LPL compared with germ-free rats, among both HC-fed and cereal-fed animals. Animals derived and maintained under germ-free conditions have been reported to display immune abnormalities, including a general decrease in T cell numbers (143; 144). Overall, exposure to cereal antigens and/or microbes stimulated increased T cell infiltration in the jejunum of BBdp rats.

Effect of diet and microbes on $CD8\alpha^+$ T cell distribution

The distribution of $CD8\alpha^+$ cells in the epithelium and lamina propria was also evaluated (**Fig. 3.2D**). The numbers of $CD8\alpha^+$ IEL and $CD8\alpha^+$ LPL were greatest in high-antigen SPF/Cereal rats. Under SPF conditions, rats fed a cereal diet had significantly increased numbers of both $CD8\alpha^+$ IEL (**Fig. 3.2E**) and $CD8\alpha^+$ LPL (**Fig. 3.2F**) compared with HC-fed rats. Microbial status also influenced $CD8\alpha^+$ T cell distribution. The numbers of both $CD8\alpha^+$ IEL and $CD8\alpha^+$ LPL were increased in SPF/Cereal rats compared with Germ-free/Cereal counterparts. No significant microbial effect on $CD8\alpha^+$ IEL was observed among HC-fed rats, as HC-fed SPF-housed rats displayed very low numbers of these cells. However, there was a trend of increased $CD8\alpha^+$ LPL in SPF/HC rats compared with Germ-free/HC rats.

FIGURE 3.2

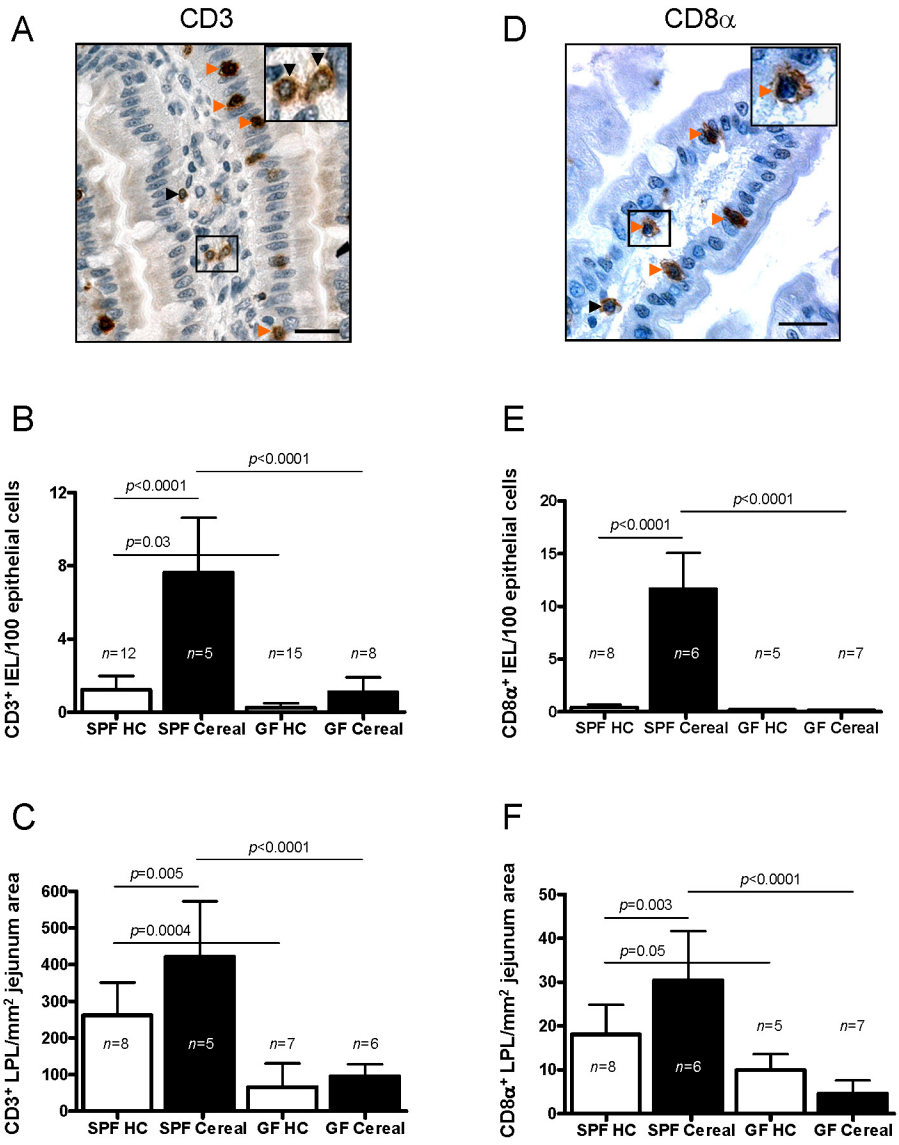

A
CD3
D
CD8α
B
CD3⁺ IEL/100 epithelial cells
p<0.0001
p<0.0001
p=0.03
n=12
n=5
n=15
n=8
SPF HC
SPF Cereal
GF HC
GF Cereal
E
CD8α⁺ IEL/100 epithelial cells
p<0.0001
p<0.0001
n=8
n=6
n=5
n=7
C
CD3⁺ LPL/mm² jejunum area
p<0.0001
p=0.005
p=0.0004
n=8
n=5
n=7
n=6
F
CD8α⁺ LPL/mm² jejunum area
p<0.0001
p=0.003
p=0.05
n=8
n=6
n=5
n=7

Figure 3.2. Effect of diet and microbes on T cell distribution
Small intestinal $CD3^+$ T cell infiltration was evaluated in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). (**A**) Representative image displaying immunostaining of CD3 in jejunum of cereal-fed BBdp rat; bar=20 μm. $CD3^+$ IEL and LPL are indicated by orange and black arrows, respectively. Inset shows digital magnification of boxed region. Effect of environment on (**B**) density of $CD3^+$ IEL and (**C**) number of $CD3^+$ LPL. (**D**) Representative image displaying immunostaining of CD8α in jejunum of cereal-fed BBdp rat; bar=20 μm. $CD8\alpha^+$ IEL and LPL are indicated by orange and black arrows, respectively. Inset shows digital magnification of boxed region. Effect of environment on (**E**) density of $CD8\alpha^+$ IEL and (**F**) number of $CD8\alpha^+$ LPL. *n*=5-15 rats/group. Data represent mean ± SD. *P*-values obtained using ANOVA followed by LSD post-hoc test. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

Overall, the distribution of $CD3^+$ and $CD8\alpha^+$ cells in the jejunum was similarly modified by environmental alteration. The combination of microbes and cereal exposure was associated with the largest numbers of gut-infiltrating $CD3^+$ and $CD8\alpha^+$ T cells. Conversely, low-antigen combinations of germ-free housing and/or HC feeding resulted in the inhibition of jejunal T cell infiltration. There were significant positive correlations between $CD3^+$ and $CD8\alpha^+$ IEL and LPL (data not shown), indicating that environmental alteration of T cell infiltration patterns was coordinated among the T cell subsets.

Effect of diet and microbes on regulatory $Foxp3^+$ T cell distribution

Expression of the master transcription factor Foxp3 specifies a major subset of anti-inflammatory $CD4^+CD25^+$ regulatory T cells ($Foxp3^+$ T_{reg}), which prevent autoimmunity and inhibit chronic inflammation (145). Nuclear staining of Foxp3 was performed and labeled cells were localized exclusively to the lamina propria (**Fig. 3.3A**). Double immunofluorescence staining confirmed nuclear Foxp3 co-localization with CD3 cell surface expression in a subset of LPL (not shown). The largest number of $Foxp3^+$ cells was present in low-antigen Germ-free/HC rats (**Fig. 3.3B**). However, no significant diet- or microbe-associated differences in $Foxp3^+$ cells were observed. Because total $CD3^+$ cell number in the lamina propria displayed significant differences associated with diet and microbial status (**Fig. 3.2C**), T_{reg} were also quantified in relation to $CD3^+$ LPL as reported by our group and others (103; 146; 147). Using this normalization approach, diet had a significant effect under germ-free conditions, with cereal-fed rats displaying a lower $Foxp3^+$ LPL/$CD3^+$ LPL T_{reg} ratio compared with HC-fed rats (**Fig. 3.3C**). However, diet did not modify the distribution of $Foxp3^+$ T_{reg} in standard SPF housing conditions. Microbial status had a significant effect

on the proportion of Foxp3$^+$ T_{reg}. Among HC-fed rats, a significantly increased Foxp3$^+$ LPL/CD3$^+$ LPL T_{reg} ratio was observed in germ-free rats compared with SPF-housed rats. Overall, significant negative correlations were observed among the proportion of Foxp3$^+$ T_{reg} and the numbers of both CD3$^+$ IEL (**Fig. 3.3D**) and CD3$^+$ LPL (**Fig. 3.3E**), indicating T_{reg} inhibition of total T cell infiltration.

Distribution of CD3$^+$ LPL and Foxp3$^+$ T_{reg} in control BBc rats, asymptomatic BBdp rats and overt diabetic BBdp rats

To expand the analysis of T_{reg} in the BBdp jejunum, CD3$^+$ LPL and Foxp3$^+$ LPL were quantified in control (diabetes-resistant) BBc rats, asymptomatic BBdp rats, and overt diabetic BBdp rats. Animals in this analysis were all SPF-housed and cereal-fed. The number of CD3$^+$ LPL was significantly increased in BBc rats compared with both asymptomatic BBdp rats and overt diabetic BBdp rats, consistent with the presence of lymphopenia in these animals (**Appendix A.2A**). The number of Foxp3$^+$ T_{reg} in overt diabetic BBdp rats (normalized to mucosal area) was significantly increased compared with asymptomatic BBdp rats (**Appendix A.2B**). The Foxp3$^+$ LPL/CD3$^+$ LPL T_{reg} ratio was significantly increased in overt diabetic BBdp rats compared with either BBc rats or asymptomatic BBdp rats and was similar between BBc and asymptomatic BBdp rats (**Appendix A.2C**). Thus, a small intestinal deficit in Foxp3$^+$ T_{reg} was not a feature distinguishing asymptomatic BBdp rats from BBc rats. Taken together with the lack of dietary modification of Foxp3$^+$ cells in SPF-housed rats (**Figs. 3.3B-C**), we concluded that a deficit in adaptive immune regulation was not a feature of the gut immune infiltrate that reflected conditions associated with T1D promotion.

FIGURE 3.3

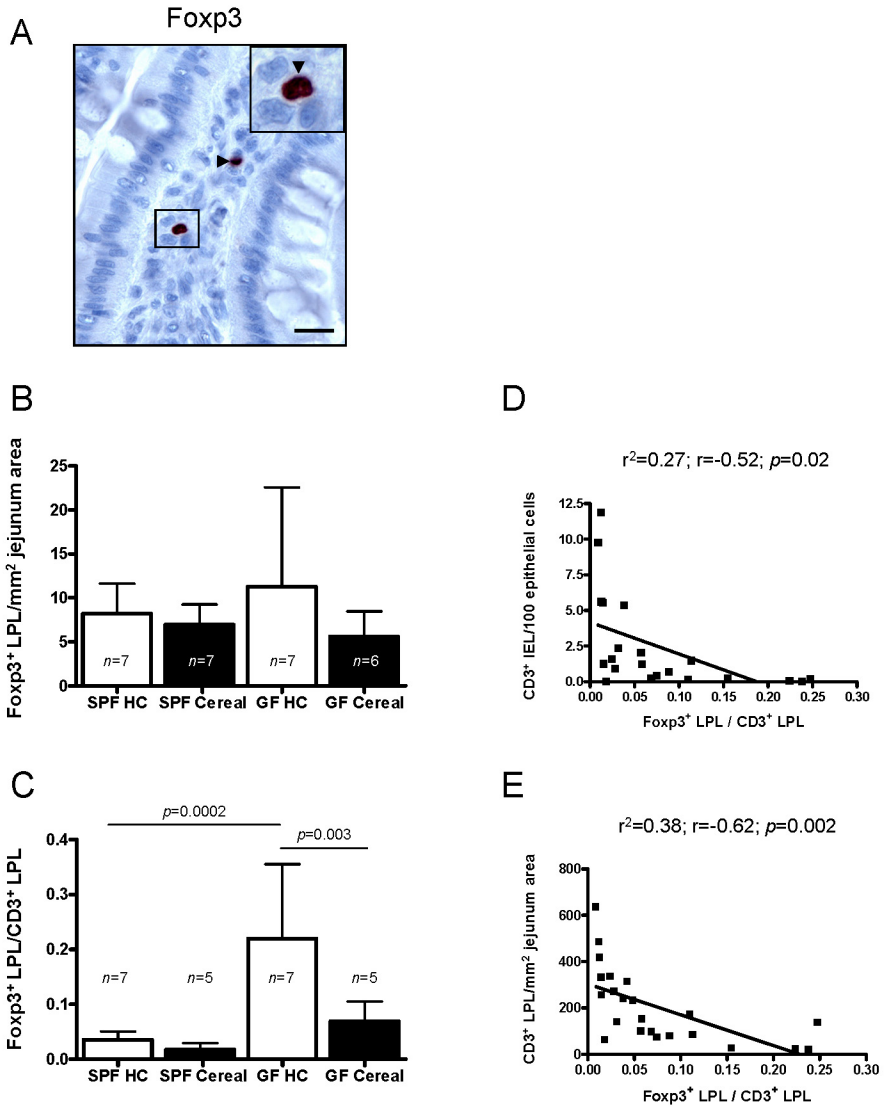

A
Foxp3
B
Foxp3+ LPL/mm2 jejunum area
n=7
n=7
n=7
n=6
SPF HC
SPF Cereal
GF HC
GF Cereal
C
Foxp3+ LPL/CD3+ LPL
p=0.0002
p=0.003
n=7
n=5
n=7
n=5
SPF HC
SPF Cereal
GF HC
GF Cereal
D
r2=0.27; r=-0.52; p=0.02
CD3+ IEL/100 epithelial cells
Foxp3+ LPL / CD3+ LPL
E
r2=0.38; r=-0.62; p=0.002
CD3+ LPL/mm2 jejunum area
Foxp3+ LPL / CD3+ LPL

Figure 3.3. Effect of diet and microbes on regulatory Foxp3$^+$ T cell distribution
The distribution of regulatory Foxp3$^+$ T cells was evaluated in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). (**A**) Representative image displaying nuclear expression of Foxp3 in jejunum of cereal-fed BBdp rat; bar=20 μm. Arrows indicate labeled cells; inset shows digital magnification of boxed region. Effect of environment on (**B**) number of Foxp3$^+$ LPL/mm^2 mucosal area and (**C**) the proportion of Foxp3$^+$ cells normalized to the number of CD3$^+$ LPL (Foxp3$^+$/CD3$^+$ ratio). *n*=5-7 rats/group. Data represent mean ± SD. P-values obtained using ANOVA followed by LSD post-hoc test. (**D**) Negative correlation between the proportion of Foxp3$^+$ cells and the density of CD3$^+$ IEL in jejunum of 130 d asymptomatic BBdp rats. (**E**) Negative correlation between the proportion of Foxp3$^+$ cells and the number of CD3$^+$ LPL. *n*=22-24. Correlation coefficients obtained using Pearson's correlation; *p*-values obtained by linear regression analysis. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

Effect of diet and microbes on macrophages

CD163 is a surface scavenger receptor present on tissue-resident M2 macrophages, which display anti-inflammatory properties (111; 112; 148; 149). Labeling of $CD163^+$ cells in the jejunum revealed distribution in the lamina propria as well as a smaller subset in close association with the gut epithelium (**Fig. 3.4A**). Epithelium-associated $CD163^+$ cells often displayed elongated pseudopodia that extended across the apical portion of enterocytes directly exposed to the gut lumen. Under SPF conditions, the number of $CD163^+$ cells was significantly increased in BBdp rats fed a protective HC diet compared with a cereal diet (**Fig. 3.4B**). In the absence of microbes, no dietary alteration of $CD163^+$ cells was observed. Microbial exposure resulted in a significantly increased number of $CD163^+$ cells, but only among rats fed a low-antigen HC diet. Analysis of a different macrophage marker, CD68, was also performed to quantify macrophages (**Appendix A.3A**). The number of $CD68^+$ cells was not modified by either diet or microbial status (**Appendix A.3B**). CD68 is often considered a pan-macrophage marker encompassing the pro-inflammatory M1 subset. Cells expressing CD163 outnumbered $CD68^+$ cells. Thus, CD68 did not label all macrophages. Thus, environmental alteration of macrophage frequency was restricted to the anti-inflammatory M2 subset.

Effect of diet and microbes on expression of M2 macrophage-associated genes

Expression of genes encoding factors associated with anti-inflammatory $CD163^+$ M2 macrophages, *Cd163* (CD163) and *Hmox1* (heme oxygenase-1; HO-1), respectively, was evaluated. The lowest expression of *Cd163* was observed in high-antigen SPF/Cereal-fed rats (**Fig. 3.4C**). Among SPF-housed rats, HC feeding resulted in significantly increased *Cd163* expression compared with cereal feeding, consistent with morphometric quantification of

$CD163^+$ cells (**Fig. 3.4B**). Expression of *Hmox1* was highest in rats from the low-antigen Germ-free/HC group (**Fig. 3.4D**). Among HC-fed animals, the presence of microbes significantly decreased *Hmox1* expression. Among cereal-fed rats, expression of *Hmox1* was similar in SPF-housed and germ-free rats. Overall, M2-associated gene expression was upregulated in two diabetes-protective environmental conditions, with induction of *Cd163* transcription in HC-fed diet-protected rats compared with cereal-fed rats (SPF housing) and induction of *Hmox1* in Germ-free/HC rats compared with SPF/HC rats.

Associations between $CD163^+$ macrophages and other immune cells in the BBdp jejunum

As observed for $Foxp3^+$ T_{reg}, larger numbers of $CD163^+$ macrophages were associated with fewer infiltrating T cells; significant negative correlations were observed among the number of $CD163^+$ cells and the numbers of both $CD3^+$ IEL (**Fig. 3.5A**) and $CD3^+$ LPL (**Fig. 3.5B**) among SPF-housed rats. Similarly, a significant negative correlation was observed between the number of $CD163^+$ cells and the density of $CD8\alpha^+$ IEL (**Fig. 3.5C**); no significant association was observed with $CD8\alpha^+$ LPL (**Fig. 3.5D**).

Effect of diet and microbes on expression of immunomodulatory cytokine genes

Expression of *Il10* (IL-10) was smallest in low-antigen Germ-free/HC rats (**Fig. 3.6A**). Animals housed under SPF conditions displayed increased expression of *Il10* compared with germ-free rats, which was significant among HC-fed rats. There were no significant dietary differences in *Il10* expression in either SPF-housed or germ-free rats.

FIGURE 3.4

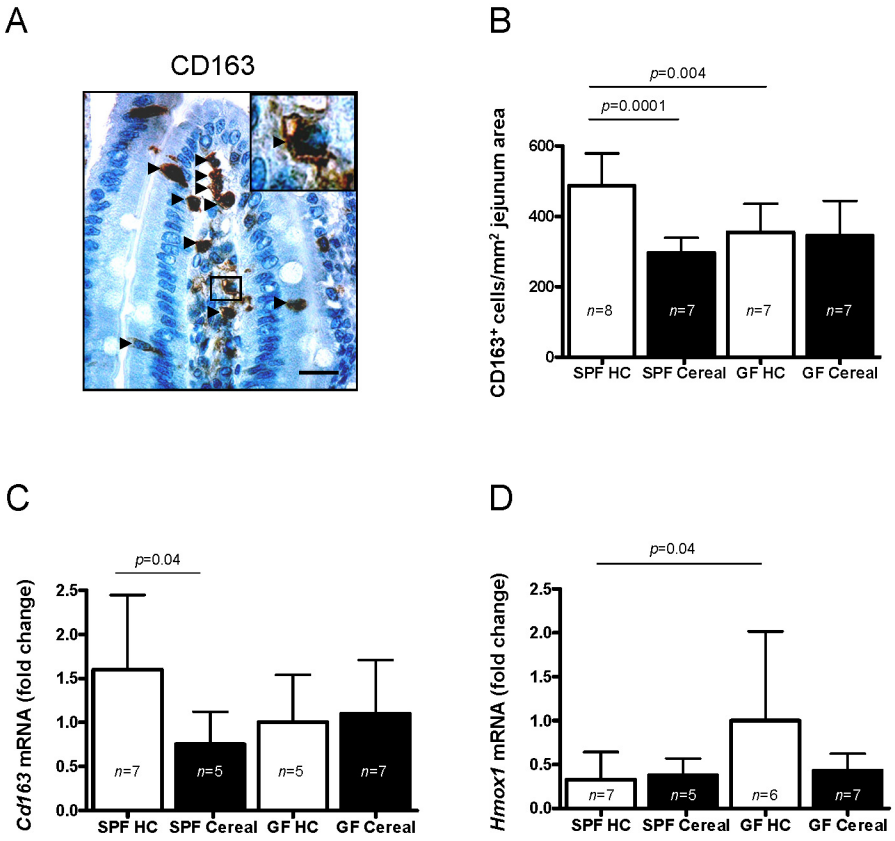

A
CD163
B
p=0.004
p=0.0001
CD163+ cells/mm2 jejunum area
600
400
200
0
n=8
n=7
n=7
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
C
p=0.04
Cd163 mRNA (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
n=7
n=5
n=5
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
D
p=0.04
Hmox1 mRNA (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal

Figure 3.4. Effect of diet and microbes on M2 macrophages and M2-associated gene expression

M2 macrophages were analyzed in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). (**A**) Representative image displaying CD163 immunostaining in jejunum of cereal-fed BBdp rat; bar=20 μm. Arrows indicate labeled cells; inset shows digital magnification of boxed region. (**B**) Number of $CD163^{+}$ cells in small intestine of BBdp rats. Jejunal mRNA expression of anti-inflammatory M2-associated innate immune genes was evaluated. Gene expression of (**C**) *Cd163* and (**D**) *Hmox1* in BBdp rat jejunum. *n*=5-8 rats/group. Data represent mean ± SD. *P*-values obtained using ANOVA followed by LSD post-hoc test. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

FIGURE 3.5

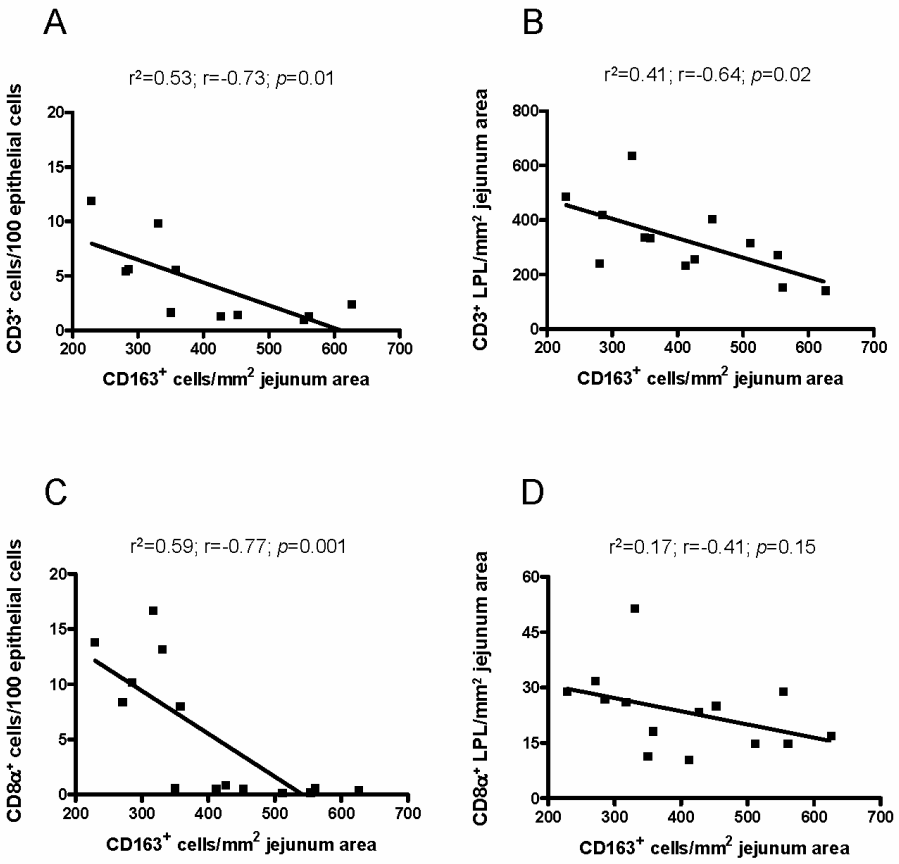

A
r²=0.53; r=-0.73; p=0.01
CD3⁺ cells/100 epithelial cells
CD163⁺ cells/mm² jejunum area
B
r²=0.41; r=-0.64; p=0.02
CD3⁺ LPL/mm² jejunum area
CD163⁺ cells/mm² jejunum area
C
r²=0.59; r=-0.77; p=0.001
CD8α⁺ cells/100 epithelial cells
CD163⁺ cells/mm² jejunum area
D
r²=0.17; r=-0.41; p=0.15
CD8α⁺ LPL/mm² jejunum area
CD163⁺ cells/mm² jejunum area

Figure 3.5. Association between CD163$^+$ M2 macrophages and total infiltrating T cells
Negative correlations between the number of CD163$^+$ cells and (**A**) the density of CD3$^+$ IEL and (**B**) the number of CD3$^+$ LPL (SPF condition). (**C**) Negative correlation between the number of CD163$^+$ cells and the density of CD8α^+ IEL (SPF condition); (**D**) lack of significant association between CD163$^+$ cells and CD8α^+ LPL (SPF condition). *n*=11-14. Correlation coefficients obtained using Pearson's correlation; *p*-values obtained by linear regression analysis.

SPF-housed BBdp rats displayed increased expression of *Tgfb1* (TGF-β1) compared with germ-free rats, which was significant among HC-fed rats (**Fig. 3.6B**). Consistent with *Il10*, no significant diet-associated differences in *Tgfb1* expression were observed. Cereal feeding induced *Il4* (IL-4) expression compared with HC feeding under germ-free conditions (**Fig. 3.6C**). The transcription pattern of *Tgfb1* was consistent with *Il10* data and expression of these cytokines was positively correlated (data not shown).

Effect of diet and microbes on expression of pro-inflammatory cytokine genes

Expression of *Ifng* (IFN-γ) was lowest in rats from the low-antigen Germ-free/HC group compared with all other groups (**Fig. 3.7A**), demonstrating inhibition of pro-inflammatory T_H1 transcription in the absence of diabetes-promoting environmental antigens. Under germ-free conditions, significantly increased *Ifng* expression was observed in cereal-fed rats compared with HC-fed rats. Also, SPF-housed animals displayed significantly increased *Ifng* expression compared with germ-free rats among those fed an HC diet. Interestingly, no microbial difference was observed among cereal-fed rats, indicating that cereal antigens were sufficient for induction of *Ifng* gene expression in the jejunum. Cereal and microbes both induced *Ifng* transcription when either was the principal source of environmental antigens. Expression of *Il15* (IL-15) was significantly increased in cereal-fed rats compared with HC-fed animals in the absence of microbes (**Fig. 3.7B**). Expression of *Il15* was also increased under germ-free conditions compared with SPF conditions among rats fed a cereal-based diet; no microbe-associated difference was observed among HC-fed rats. No significant diet- or microbe-associated changes in *Il17a* (IL-17A) expression were observed (**Fig. 3.7C**). Expression of another T_H17-associated cytokine gene, *Il23* (IL-23), was increased in SPF

conditions compared with germ-free conditions among rats fed an HC diet; no other significant environmental differences were observed (**Fig. 3.7D**).

Effect of diet and microbes on jejunal T_H gene expression bias

Cytokine gene expression ratios were determined for evaluation of the effect of environmental exposures on T_H transcriptional bias in the BBdp jejunum. Cytokine gene expression ratios were determined for T_H1-associated *Ifng* expression in relation to counter-regulatory T_H2- and T_{reg}-associated cytokines: *Ifng*/*Il4*, *Ifng*/*Tgfb1*, and *Ifng*/*Il10*. The T_H1/T_H2 transcription ratio of *Ifng*/*Il4* was highest in high-antigen SPF/Cereal rats and lowest in low-antigen Germ-free/HC rats (**Fig. 3.8A**), reflecting high and low extremes of T1D incidence, respectively. Among SPF-housed animals, cereal feeding was associated with a significantly increased *Ifng*/*Il4* expression ratio, indicating T_H1 transcriptional bias compared with HC feeding. Among cereal-fed rats, microbe exposure promoted an increased *Ifng*/*Il4* expression ratio compared with the germ-free condition; no significant microbe-associated difference was observed among HC-fed rats. The ratio of *Ifng*/*Il10* was significantly increased in cereal-fed rats compared with HC-fed under germ-free conditions (**Fig. 3.8B**). Similarly, the expression ratio of *Ifng*/*Tgfb1* was significantly increased in cereal-fed rats compared with HC-fed rats in the absence of microbes (**Fig. 3.8C**). There were trends of increased cereal-associated *Ifng*/*Il17a* expression under both SPF conditions and germ-free conditions but the differences were not statistically significant (**Fig. 3.8D**). Overall, in the presence or absence of microbes, expression of *Ifng* predominated over expression of counter-regulatory T_H2- and T_{reg}-associated cytokine genes in animals fed cereal diets, suggesting T_H1 transcriptional bias relative to HC-fed counterparts.

FIGURE 3.6

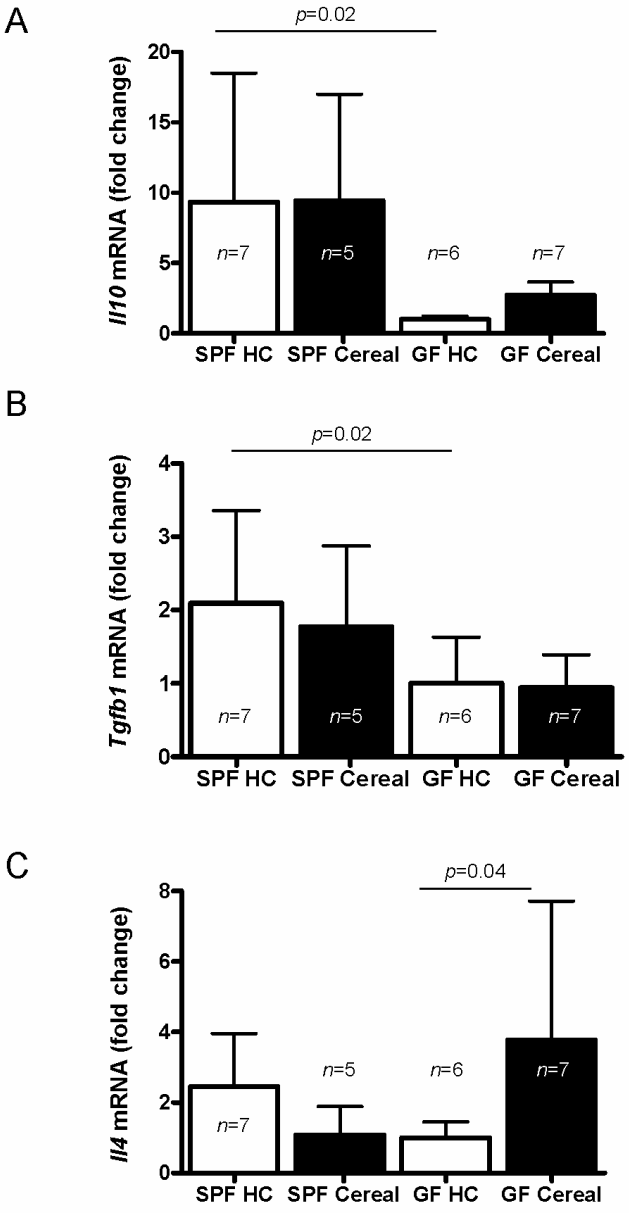

A
p=0.02
Il10 mRNA (fold change)
20
15
10
5
0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
B
p=0.02
Tgfb1 mRNA (fold change)
4
3
2
1
0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
C
p=0.04
Il4 mRNA (fold change)
8
6
4
2
0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal

Figure 3.6. Effect of diet and microbes on expression of immunomodulatory cytokine genes

Small intestinal mRNA expression of immunomodulatory T_H2- or T_{reg}- associated cytokines *Il10*, *Tgfb1*, and *Il4* was evaluated in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). Gene expression of (**A**) *Il10*, (**B**) *Tgfb1*, and (**C**) *Il4*. *n*=5-7/group. Data represent mean ± SD. Fold change values are presented relative to the low-antigen Germ-free/HC group. *P*-values obtained using ANOVA followed by LSD post-hoc test. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

FIGURE 3.7

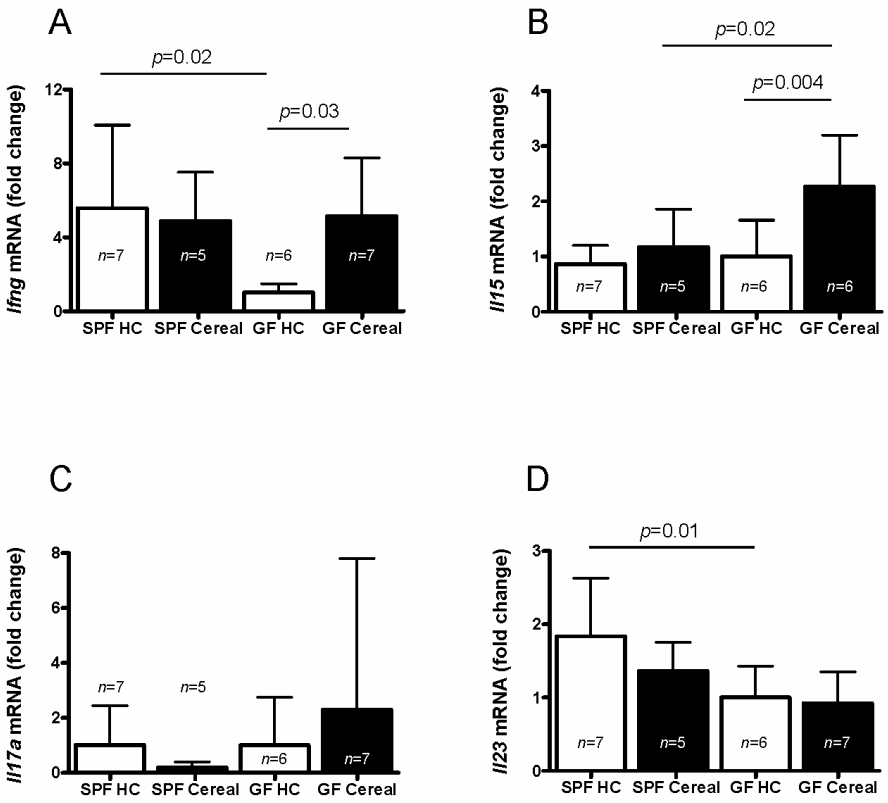

A
p=0.02
p=0.03
Ifng mRNA (fold change)
12
8
4
0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
B
p=0.02
p=0.004
Il15 mRNA (fold change)
4
3
2
1
0
n=7
n=5
n=6
n=6
SPF HC
SPF Cereal
GF HC
GF Cereal
C
Il17a mRNA (fold change)
8
6
4
2
0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
D
p=0.01
Il23 mRNA (fold change)
3
2
1
0
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal

Figure 3.7. Effect of diet and microbes on expression of pro-inflammatory cytokine genes

Small intestinal mRNA expression of pro-inflammatory T_H1-associated cytokines, *Ifng* and *Il15*, was evaluated in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). Gene expression of (**A**) *Ifng*, (**B**) *Il15*, (**C**) *Il17a,* and (**D**) *Il23*. *n*=5-7/group. Data represent mean ± SD. Fold change values are presented relative to the low-antigen Germ-free/HC group. *P*-values obtained using ANOVA followed by LSD post-hoc test. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

FIGURE 3.8

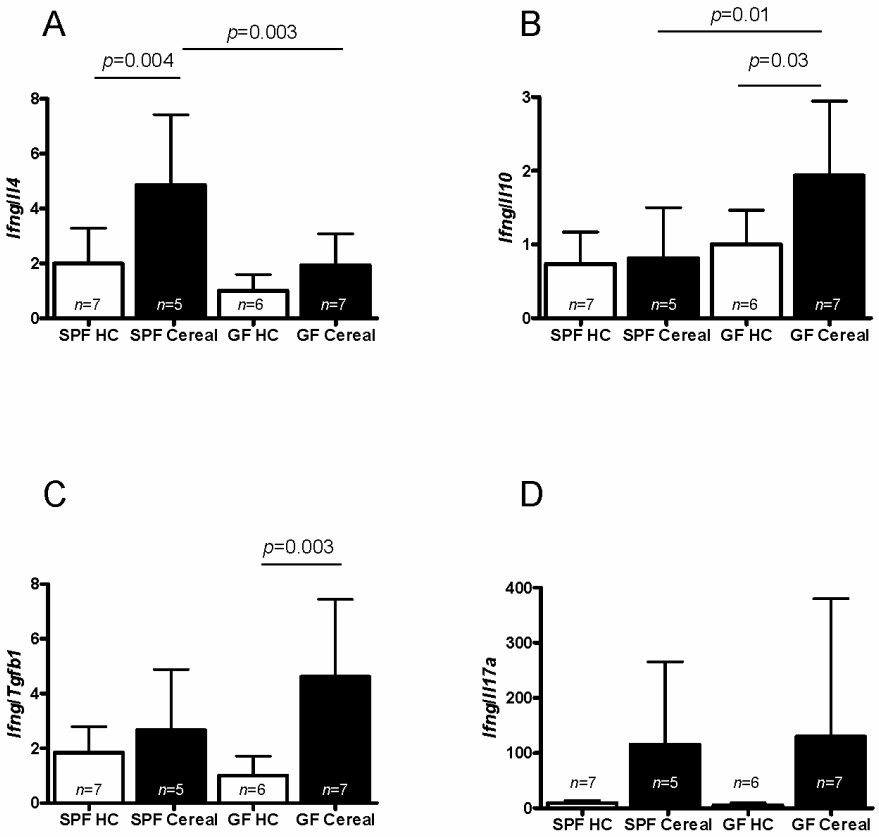

A
p=0.004
p=0.003
Ifng/Il4
n=7
n=5
n=6
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal
B
p=0.01
p=0.03
Ifng/Il10
C
p=0.003
Ifng/Tgfb1
D
Ifng/Il17a

Figure 3.8. Effect of diet and microbes on jejunal T_H gene expression profile
Small intestinal mRNA expression ratios of major hallmark cytokines, as a reflection of T_H transcriptional bias, were determined in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). (**A**) T_H1/T_H2 cytokine gene expression ratio of *Ifng*/*Il4* in BBdp rat jejunum. T_H1/T_{reg} cytokine gene expression ratios of (**B**) *Ifng*/*Il10* and (**C**) *Ifng*/*Tgfb1*. (**D**) T_H1/T_H17 cytokine expression ratio of *Ifng*/*Il17a*. *n*=5-7/group. Data represent mean ± SD. Fold change values are presented relative to the low-antigen Germ-free/HC group. *P*-values obtained using ANOVA followed by LSD post-hoc test. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

SUMMARY

The role of environment in T1D pathogenesis is controversial. In the present study, diet and microbes were separated to investigate their effect on T1D development in BBdp rats. Exposure to cereal antigens provided the largest diabetes-promoting stimulus, either in the presence or absence of microbes. Microbes modestly promoted T1D, but only in the absence of cereal antigens. Characterization of the small intestinal immune infiltrate was the primary focus of the study, in order to determine what immune features were related to environmental conditions that influenced T1D development. Overall, promotion of T1D by either cereal or microbes was associated with increased jejunal T cell infiltration and T_H1 transcriptional bias in conjunction with deficits in anti-inflammatory immune cells. Thus, small intestinal inflammation parallels T1D development in BBdp rats.

DISCUSSION

The diabetes-modulating potential of two major sources of ubiquitous external influences, diet and microbes, was studied in BBdp rats, with emphasis on the small intestine, an organ that integrates environment and the immune system. Unlike previous studies, the effects of diet and microbes were explored in isolation and in combination. In the presence of microbes, cereal feeding promoted increased T1D compared with HC feeding, consistent with previous work from our group and others (2; 41; 51; 52; 150). The results are also consistent with studies of NOD mice fed diets lacking wheat proteins (60; 69; 151). In the absence of microbes, cereal was also diabetes-promoting whereas HC feeding afforded near-complete protection. Thus, dietary modification of T1D was observed under both standard and sterile housing conditions, with exposure to cereal antigens sufficient for strong T1D induction without the requirement for live microbes or viruses. Development of spontaneous

T1D in germ-free BB-DP rats was previously reported by Rossini *et al*. in an older study (152); the present data confirm that finding and further reveal that dietary modification of T1D is maintained in the absence of microbes. Microbe-associated promotion of T1D was only observed in rats fed a low-antigen HC diet lacking diabetes-promoting cereal antigens. Thus, diet and microbes jointly modulated T1D pathogenesis in BBdp rats, with cereal representing the predominant environmental inducer. These results are consistent with a growing body of evidence supporting an association between cereal exposure and T1D. Prospective studies in children at risk for T1D revealed that introduction of gliadin-containing cereals was associated with increased risk of islet autoimmunity (79-81; 153). The mechanism by which cereal antigens stimulate T1D is not completely understood but could involve inflammation in the gastrointestinal tract (2). In the present study, promotion of T1D was associated with T cell infiltration in the small intestine. Under SPF conditions in the presence of microbiota, there were striking increases in the numbers of infiltrating $CD3^+$ and $CD8\alpha^+$ IEL and LPL in the jejunum of rats fed a cereal diet compared with an HC diet. Hanninen *et al*. reported that T cells originating in the intestinal mucosa were among the earliest lymphocytes to infiltrate pancreatic islets during T1D development in NOD mice (65).

Many clues to cereal-associated promotion of T1D can be obtained from our understanding of celiac disease, in which enteropathy and autoimmunity develop as a result of dietary gluten ingestion (154). As observed in celiac disease, it is likely that the major consequence of cereal protein exposure in diabetes-prone subjects is gut immune activation. Unlike many common food proteins, gluten proteins from grains such as wheat, barley, and rye are resistant to extensive proteolysis from digestive enzymes (154). In celiac-susceptible

subjects with HLA-DQ2/DQ8 haplotypes, which are also associated with T1D risk, accumulation of intact wheat-derived proteins can induce a pro-inflammatory immune response causing gut damage (154; 155).

Graham *et al*. previously reported that BBdp rats displayed an increased number of IEL compared with BBc rats (44). In the present study, cereal feeding stimulated IEL infiltration in the BBdp rat jejunum. Although microbes also promoted T cell infiltration compared with germ-free conditions, cereal feeding exacerbated T cell infiltration in the presence of microbes (**Fig. 3.2**). In a study by Maurano *et al*., feeding NOD mice a gluten-free diet inhibited T1D development in association with decreased IEL infiltration in the jejunum, consistent with the present findings in BBdp rats (60). In addition, jejunal tissue from cereal-fed NOD mice displayed increased T_H1-biased *Ifng* gene expression compared with mice fed either a gluten-free diet (60) or ProSobee (156), also consistent with the present data. A low-grade enteropathy was also described in the colon of young cereal-fed NOD mice, which did not develop in mice fed a ProSobee diet (157). Taken together, these findings indicate that cereal feeding promotes T1D in two different rodent models, which is paralleled by jejunal T cell infiltration in conjunction with pro-inflammatory T_H1-biased gene expression.

Consistent with rodent data, signs of immunological activation characterized by T_H1 inflammation have been reported in the small intestine of humans with T1D (137). Westerholm-Ormio *et al*. reported that jejunum biopsies of patients with T1D were infiltrated by increased numbers of IFN-γ^+ cells compared with non-diabetic control subjects (137).

Similarly, Savilahti *et al*. observed increased numbers of lamina propria $\alpha_4\beta_7^+$ T cells and strong HLA class II expression in jejunum biopsies from children with T1D compared with non-diabetic controls, which the authors attributed to enhanced T_H1 activation (138). Cereal exposure can exacerbate inflammation in the small intestine of patients with T1D. For example, Auricchio *et al*. reported that small intestinal biopsies from children with T1D cultured with gliadin displayed increased $CD3^+$ IEL and activated lamina propria $CD25^+$ cells compared with biopsy samples from control subjects (158). These data indicate that inflammation is present in the jejunum of patients with T1D and suggest that the small intestine of these patients is immunologically more responsive to cereal antigens. Thus, the already-impaired gastrointestinal tract of diabetes-prone subjects becomes inflamed by cereal exposure in rodent models and some patients, suggesting that gut inflammation may parallel or precede T1D development.

IEL infiltration is potentially a sensitive biomarker of cereal-associated inflammation in T1D-susceptible subjects. Patients with other diseases involving the gastrointestinal tract, including celiac disease, Crohn's disease, and ulcerative colitis feature increased IEL infiltration associated with tissue damage and gut leakiness (159-161). Increased IEL density is among the earliest markers of celiac histopathology and represents one of the most sensitive measures of cereal-associated jejunal inflammation in the context of gluten-sensitive enteropathy (162). Using human jejunum biopsies from patients with celiac disease, Leigh *et al*. reported a dose-dependent increase in IEL infiltration upon patient ingestion of pepsin-trypsin gluten digests only 12 hours after oral challenge without other signs of mucosal damage or enteropathy (162). Increased IEL infiltration serves as the only quantifiable pathological indication of celiac-associated inflammation in instances where

mucosal architecture otherwise appears normal (128; 162). Thus, IEL inflammation could represent a primary lesion associated with abnormal intestinal immune reactivity to cereal antigens in susceptible subjects. In celiac disease, the majority of IEL contain cytotoxic granules displaying an activated inflammatory phenotype (128). In the present study, $CD3^+$ cell infiltration (both IEL and LPL) paralleled extremes of T1D incidence in BBdp rats. Increased T cell infiltration in the jejunum of cereal-fed BBdp rats under standard SPF housing conditions suggests an association among diet, small intestinal T cell inflammation, and T1D development. Thus, an important mechanism of dietary modulation of T1D by low-antigen HC feeding could be directly related to inhibition of jejunal T cell infiltration.

Increased gut permeability preceding T1D onset has previously been described as an abnormality in BBdp rats (51). Evidence of gut leakiness in patients with T1D has also been reported (140; 163). Closing tight junctions in BBdp rats by blocking the zonulin/pre-HP2 pathway decreased gut permeability and inhibited T1D development in BBdp rats (56; 58). Visser *et al*. reported that HC feeding decreased small intestinal permeability in association with decreased serum zonulin/pre-HP2 concentration (52). In a prospective analysis, Sapone *et al*. reported that serum zonulin/pre-HP2 concentration was increased in the majority of susceptible subjects that progressed to T1D, suggesting that gut barrier impairments precede T1D in many humans (59). A potential mechanism by which gut leakiness can promote T1D is via stimulation of gut immune inflammation. In SPF rats, HC feeding decreased the T_H1-biased *Ifng*/*Il4* cytokine gene expression ratio and T_H1 transcription was also inhibited by HC feeding under sterile conditions. Pro-inflammatory cytokines including IFN-γ and TNF-α can directly damage the intestinal epithelium by disturbing tight junction organization

(154; 164). For example, IFN-γ has been reported to promote endocytosis of tight junction components including claudins, occludins, and junctional adhesion molecule 1 (165; 166), promoting gut leakiness (154). Small intestinal leakiness becomes apparent at ~50 d in BBdp rats (51; 58). Thus, it is possible that small intestinal inflammation promoted by cereal exposure starting at weaning (23 d) precedes (and possibly causes) gut leakiness in BBdp rats. HC feeding could exert protective effects by decreasing intestinal inflammation and leakiness in parallel, preventing pro-inflammatory immune activation.

The finding of increased jejunal T cell infiltration and T_H1 transcriptional bias in cereal-fed BBdp rats is consistent with other studies by our group. Chakir *et al*. reported that isolated MLN-derived cells from cereal-fed BBdp rats displayed an increased percentage of IFN-γ^+ T cells compared with cells from either HC-fed BBdp counterparts or cereal-fed BBc rats (36). In addition, Mojibian *et al*. found that $CD3^+$ T cells from patients with T1D proliferated upon exposure to chymotrypsin-digested wheat gluten (82). Wheat peptide exposure stimulated a mixed pro-inflammatory cytokine response in PBMCs from patients that was characterized by pronounced IFN-γ secretion (82).

The association among diet, intestinal microbes, and T1D was previously studied in BB-DP rats by Brugman *et al*. and found to be similar to the present study (89). In antibiotic-treated BB-DP rats fed a cereal diet, T1D incidence was ~38% compared with 86% in antibiotic-free controls (89). Most strikingly, a combination of antibiotic treatment and low-antigen HC feeding abrogated T1D in all animals whereas HC feeding without antibiotics resulted in T1D incidence of 53% (89). Consistent with those results, Hansen *et al*. reported

that feeding NOD mice a gluten-free diet inhibited T1D in association with decreased caecal bacteria (167). These findings are in keeping with the present results, notably the additive diabetes-promoting combination of cereal and microbes (89). As Brugman *et al*. pointed out, intestinal "antigenic load" was likely a key determinant of T1D development in BB-DP rats and the present results support this interpretation (89). Overall, combinations of decreased microbes and low-antigen HC feeding afforded dramatic protection from T1D in BBdp rats (89). The present study definitively ascribes a primary role for cereal antigens in T1D development, as microbes were completely absent rather than merely reduced using antibiotics.

Although not required for spontaneous T1D (**Fig. 3.1A** (103); (152)), microbes exhibited diabetes-promoting capacity, but only in the absence of cereal antigens (e.g. SPF/HC vs. Germ-free/HC). There are several reports of altered microbiota associated with T1D in rodent models. Roesch *et al*., reported that fecal bacteria from diabetes-resistant BB-DR rats were different compared with BB-DP samples (87). Bacterial communities were retrospectively characterized in fecal samples from young BB-DP rats in the period preceding islet inflammation (89). Rats that eventually progressed to T1D contained an increased proportion of *Bacteroides* compared with rats that remained asymptomatic (89), demonstrating that early differences in intestinal microbes were associated with different outcomes. Microbial involvement in NOD mice has also been reported. In an experiment by Funda *et al*., germ-free conditions were associated with increased T1D compared with SPF-housed mice (168). Interestingly, *Myd88*-deficient NOD mice were protected from T1D under SPF conditions but incidence was dramatically increased under germ-free conditions, underscoring the complexity of the interactions occurring between microbes and innate

immunity that influence T1D outcome (169). A low frequency of T1D in NOD mice was associated with the transmission of segmented filamentous bacteria (170). In another study, weekly LPS injections in young NOD mice resulted in near-complete inhibition of T1D (171). These reports are consistent with the "hygiene hypothesis", which proposes that improved hygiene practices leading to decreased immune stimulation by (tolerizing) environmental determinants (e.g. microbes, viruses) can stimulate chronic inflammation, allergy, and autoimmunity (172; 173). However, the only significant microbe-associated effect in BBdp rats was modest promotion of T1D rather than protection, the opposite of what would be predicted by the "hygiene hypothesis".

Other studies in NOD mice have not ascribed major roles for microbes in T1D pathogenesis. In a study by Alam *et al*., T1D incidence was equivalent in cereal-fed germ-free and SPF mice, although increased insulitis was observed under sterile conditions (174). Also, King *et al*. reported that cereal-fed germ-free NOD mice displayed similar T1D incidence compared with SPF counterparts (175). In the present study, T1D incidence was not significantly different between SPF/Cereal rats and Germ-free/Cereal rats (**Fig. 3.1A**); therefore, our findings are consistent with the aforementioned reports (174; 175). Interestingly, cultured MLN cells from young NOD mice, but not BALB/c mice, proliferated *in vitro* upon exposure to autologous fecal bacteria, suggesting decreased tolerance to commensal microbes in the diabetes-prone strain (157). Consistent with this concept, the present findings indicate that SPF/HC rats displayed increased T cell infiltration and *Ifng* expression compared with Germ-free/HC rats, which could have stimulated microbe-induced T1D. Overall, in cereal-fed diabetes-prone rodents, T1D incidence was similar among germ-

free and SPF-housed animals. Thus, gut microbes are not essential for spontaneous T1D in BBdp rats and NOD mice.

Foxp3$^+$ T_{reg} are critical for the inhibition of autoimmunity (93; 176). Foxp3$^+$ T_{reg} abnormalities have been reported in humans with T1D (97-99) and other autoimmune diseases (97; 177). Lahl *et al*. reported that Foxp3$^+$ T_{reg} depletion in newborn mice stimulated insulitis (178). Also, deficits in Foxp3$^+$ T_{reg} have been reported in the gastrointestinal tract of diabetes-prone rodents compared with control strains (46; 157). In the present study, we investigated whether protective HC feeding could inhibit T1D in part by enhancing the number of T_{reg} in the jejunum. No dietary differences in either the frequency or proportion of Foxp3$^+$ T_{reg} were observed in BBdp rats housed under SPF conditions (**Figs. 3.3B-C**). Thus, by this measure, adaptive immune regulation was not diet-modifiable in the jejunum and could not explain HC-associated dietary protection from T1D under standard microbe-exposed conditions. A similar conclusion was made in a study by Visser *et al*., as no difference in the number of Foxp3$^+$ T_{reg} was observed in MLN from cereal-fed BB-DP rats compared with HC-fed rats (46). In colonic tissue of NOD mice, Foxp3$^+$ cells were increased in cereal-fed rats in parallel with increased IFN-γ^+ cells compared with diet-protected ProSobee-fed animals (157). Collectively, these findings indicate that numbers of intestinal T_{reg} are either unchanged by diabetes-modifying diets or increased concomitantly with inflammation. What is common among these various reports is that diabetes-protective diets did not enhance T_{reg} numbers in the gastrointestinal tract of diabetes-prone rodents. However, there was a significant decrease in the proportion of Foxp3$^+$ T_{reg} in cereal-fed rats compared with HC-fed rats under germ-free conditions, which could have contributed to T1D development under sterile conditions. Also, the proportion of Foxp3$^+$ T_{reg} was increased

in Germ-free/HC rats compared with SPF/HC counterparts, which could have provided anti-inflammatory protection from microbe-induced T1D. Among cereal-fed rats, there was no microbe-associated difference in the proportion of $Foxp3^+$ T_{reg}. Consistent with this finding, Min *et al*. reported that an absence of intestinal microbes did not change the generation, accumulation, or maintenance of T_{reg} in germ-free mice compared with microbe-exposed controls (179). Under standard housing conditions, diet did not modify small intestinal T_{reg} and among rats fed a standard cereal diet, microbes did not modify T_{reg}.

$CD163^+$ M2 macrophages produce immunomodulatory cytokines, lack expression of co-stimulatory molecules, and contribute to the maintenance of a hyporesponsive immunological state (148). The number of anti-inflammatory $CD163^+$ M2 macrophages was significantly smaller in the jejunum of cereal-fed BBdp rats compared with HC-fed counterparts under standard SPF conditions (**Fig. 3.4B**). These results were further reflected by upregulation of tolerogenic *Cd163* gene expression in diet-protected HC-fed animals compared with cereal-fed (**Fig. 3.4C**). These findings indicate for the first time that M2 macrophages are modified by diet in the small intestine of diabetes-prone rats. In addition to the dietary difference among BBdp rats, our group reported a deficit in jejunal $CD163^+$ cells in BBdp rats compared with BBc animals (103). Taken together, these findings reveal a deficit in gut-resident M2 macrophages in the small intestine of BBdp rats that can be corrected by weaning onto a diabetes-protective HC diet. Thus, enhanced anti-inflammatory function contributed by increased $CD163^+$ M2 macrophages could represent a major immunomodulatory effect of diabetes-protective HC feeding. In a study by Zheng *et al*., adoptive transfer of M2 macrophages protected BALB/c mice from STZ-induced islet damage in association with decreased blood glucose compared with either control STZ-

diabetic mice or mice injected with non-polarized 'M0' cells (180). The most compelling functional evidence to date demonstrating the potential of anti-inflammatory M2 macrophages to modulate spontaneous T1D was reported by Parsa *et al*. (102). In NOD mice, M1 macrophages were manipulated *ex vivo* towards an M2-like phenotype by incubation with immunomodulatory cytokines IL-4, IL-10, and TGF-β, which inhibited production of pro-inflammatory mediators NO, IL-6, TNF-α (102). When these induced anti-inflammatory M2 macrophages were injected into young NOD mice, T1D was inhibited (102). Similarly, expansion of $CD163^+$ macrophages afforded by HC feeding beginning at weaning could inhibit immune activation and T1D development in BBdp rats. In support of this view, there were negative correlations between the numbers of $CD163^+$ cells and gut-infiltrating T cells (**Figs. 3.5A-C**). The present data support a model whereby HC feeding (or cereal avoidance) enables M2 macrophage enrichment and T1D protection in BBdp rats.

The enzyme HO-1, encoded by *Hmox1*, is critical for the degradation of heme and is essential for cytoprotection, host defense, and regulation of inflammation (181). The CD163 receptor is essential for internalization of heme substrates in the context of hemoglobin-haptoglobin complexes and HO-1 is rate-limiting for the breakdown of heme (149). The most protective low-antigen Germ-free/HC setting was associated with the strongest induction of *Hmox1* expression (**Fig. 3.4D**). This result raises the possibility that stimulation of intestinal *Hmox1* expression could have contributed to inhibition of microbe-promoted T1D in Germ-free/HC vs. SPF/HC rats. Chemical induction of HO-1 by injections of cobalt protoporphyrin (CoPP) inhibited inflammation in the *Il10*-deficient mouse model of microbe-associated colitis, an effect associated with decreased IL-12 production by colonic M1 macrophages (182). The anti-colitic effect of HO-1 induction was associated with decreased bacterial

dissemination into MLN, a protective effect attributed to enhanced macrophage bactericidal function and restoration of intestinal immune homeostasis (182). With respect to T1D, CoPP administration induced HO-1 in the pancreas of NOD mice, resulting in decreased insulitis, increased β-cell survival, and decreased blood glucose concentration (183; 184). Our group also performed a study in which HO-1 induction by CoPP inhibited T1D in BBdp rats in association with decreased gut inflammation (Husseini *et al*., unpublished results). Thus, HO-1 induction is associated with anti-inflammatory effects in the gastrointestinal tract and participates in suppression of islet autoimmunity in diabetes-prone settings.

CONCLUSION

In summary, a cereal-based diet promoted T1D in BBdp rats, either in the presence or absence of microbes. T cell infiltration was a major feature of cereal feeding under SPF conditions. In parallel, deficits in innate and adaptive anti-inflammatory immune cell subsets, including M2 macrophages and $Foxp3^+$ T_{reg}, were observed in cereal-fed animals. In SPF rats (**Fig. 3.9A**), HC feeding supported a larger number of anti-inflammatory $CD163^+$ M2 macrophages in association with decreased T cell infiltration and lower T_H1-biased gene expression. In germ-free rats (**Fig. 3.9B**), HC feeding increased the proportion of $Foxp3^+$ T_{reg} and decreased T_H1-associated transcriptional bias. Similar to cereal promotion of T1D, microbe-induced T1D also featured increased T cell infiltration and T_H1 bias (**Fig. 3.10**). These features represented major similarities between cereal-promoted T1D and microbe-promoted T1D in BBdp rats. Protection from microbe-associated T1D afforded by the sterile state was associated with an increased proportion of $Foxp3^+$ T_{reg} and induction of anti-inflammatory *Hmox1* transcription, similar to enhancement of tolerogenic factors in HC-associated dietary protection. Thus, diet and microbes jointly shaped the gut immune

phenotype in ways that reflected environmental modification of T1D incidence (**Fig. 3.1A**). Small intestinal imbalances among pro-inflammatory and anti-inflammatory innate and adaptive immune factors could represent critical aspects of T1D pathogenesis.

FIGURE 3.9

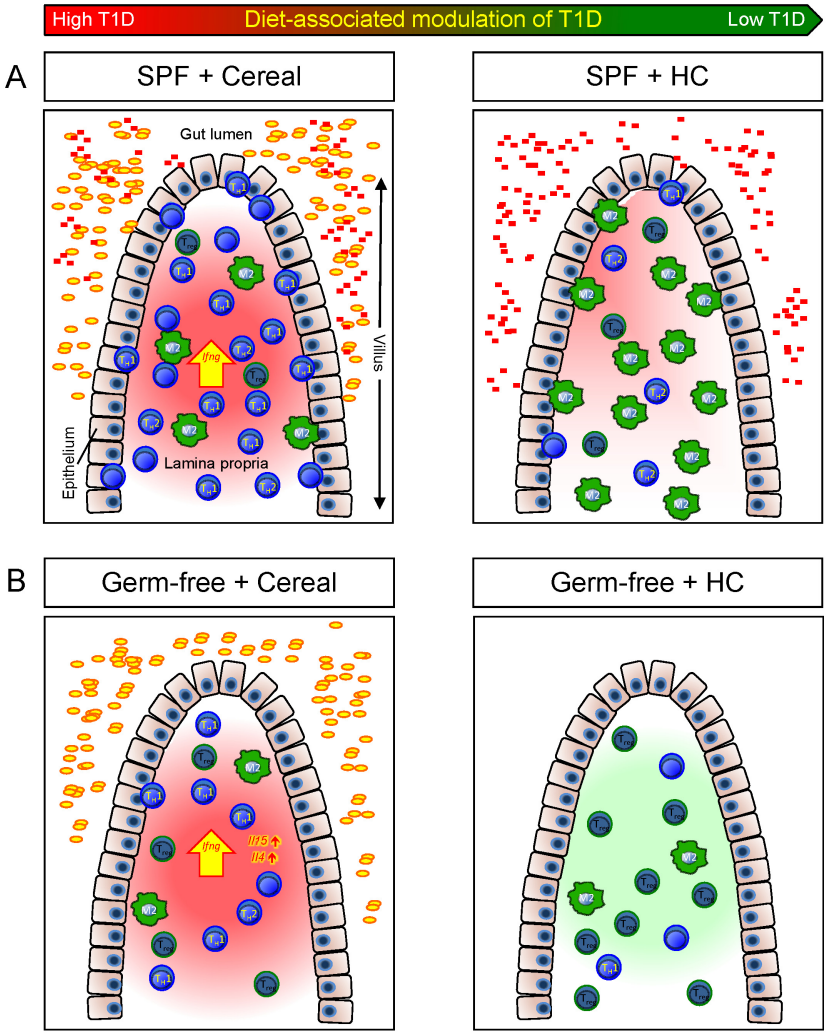

High T1D
Diet-associated modulation of T1D
Low T1D
A
SPF + Cereal
SPF + HC
Gut lumen
Villus
Epithelium
Lamina propria
Ifng
B
Germ-free + Cereal
Germ-free + HC
Il15
Il4

Figure 3.9. Overview of dietary modification of gut immune profiles in BBdp rats
Simplified diagrams indicating the major dietary modifications of immune cell distribution and immunological gene expression in the jejunum of 130 d asymptomatic BBdp rats fed cereal or HC under SPF or germ-free conditions; differences associated with alteration of T1D incidence are emphasized. (**A**) In the SPF condition, diabetes-protective HC feeding was associated with decreased T cell infiltration in the epithelium and lamina propria. In parallel, HC feeding promoted an increased number of anti-inflammatory M2 macrophages. Increased T1D risk in cereal-fed rats was associated with T_H1-associated transcriptional bias relative to protected HC-fed counterparts. (**B**) In the germ-free state, diabetes-protective HC feeding promoted an expansion in the proportion of anti-inflammatory $Foxp3^+$ T_{reg}. Consistent with the SPF condition, cereal feeding under germ-free conditions was associated with T_H1-biased transcription. Thus, in the BBdp rat jejunum, exposure to diabetes-promoting cereal antigens was characterized by deficits in anti-inflammatory immune cell subsets in conjunction with increased T cell infiltrates in the context of a T_H1-biased transcriptional milieu. Character legend: *Antigens*. Yellow ovals: cereal proteins; red rectangles: microbes. *Immune cells*. Blue: Effector T cells; green: $CD163^+$ M2 macrophages; teal: $Foxp3^+$ T_{reg}. Major transcriptional differences between conditions indicated in yellow-highlighted text.

FIGURE 3.10

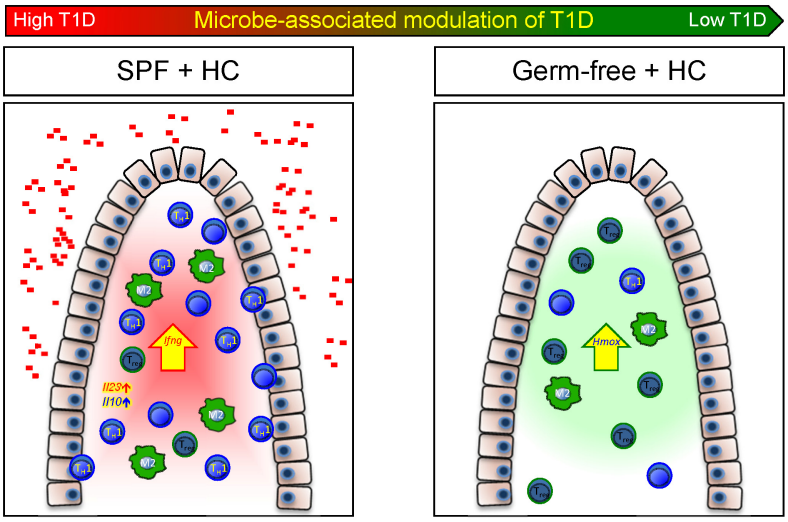

High T1D
Microbe-associated modulation of T1D
Low T1D
SPF + HC
Germ-free + HC
Ifng
Il23↑
Il10↑
Hmox

Figure 3.10. Overview of microbial modification of gut immune profile in BBdp rats
Simplified diagram indicating the major microbe-associated modifications of immune cell distribution and immunological gene expression in the jejunum of 130 d asymptomatic BBdp rats fed an HC diet under SPF or germ-free conditions; differences associated with alteration of T1D incidence are emphasized. Microbe-associated promotion of T1D was linked with a relative deficit in the proportion of anti-inflammatory $Foxp3^+$ T_{reg} compared with protective sterile conditions. Microbe-associated T1D promotion also featured increased numbers of infiltrating T cells in the jejunum. Similar to cereal-associated T1D promotion, microbe-associated promotion of T1D was associated with enhanced T_H1 transcriptional bias. The protective germ-free condition featured enhancement of anti-inflammatory *Hmox1* transcription. Thus, microbe-associated T1D promotion was reflected by a deficit in anti-inflammatory immune cells, increased T cell infiltration, and enriched T_H1-associated gene expression. Character legend: *Antigens*. Red rectangles: microbes. *Immune cells*. Blue: Effector T cells; green: $CD163^+$ M2 macrophages; teal: $Foxp3^+$ T_{reg}. Major transcriptional differences between conditions indicated in yellow-highlighted text.

CHAPTER 4. Alteration of gut immune infiltrate parallels autoimmune diabetes in LEW-DP rats

ABSTRACT

Background: The diabetes-prone Lewis (LEW.1AR1.Ztm-*iddm*, "LEW-DP") rat is a non-lymphopenic model of spontaneous T1D that occurs equally in males and females (42). The role of the gastrointestinal tract or diet in T1D development has not been described in these animals.

Methods: Jejunum samples from 45 d LEW-DP (cereal- or HC-fed) and control LEW-C rats (cereal-fed) were evaluated to determine the effects of strain and diet on small intestinal immune cell distribution and immune-associated gene expression using immunohistochemistry, morphometry, and RT-qPCR.

Results: T1D incidence was higher in cereal-fed compared with HC-fed LEW-DP rats. *Strain effects*: Pre-insulitic LEW-DP rats displayed a deficit in anti-inflammatory $CD163^+$ M2 macrophages. In addition, the number of $Foxp3^+$ T_{reg} in lamina propria and *Foxp3* gene expression were lower in jejunum of LEW-DP rats. LEW-DP rats had a deficit in intra-epithelial and lamina propria T cells but displayed an increased *Ifng*/*Tgfb1* cytokine mRNA ratio. *Dietary effects among LEW-DP rats*: In cereal-fed rats, the proportion of $Foxp3^+$ T_{reg} was smaller compared with HC-fed rats. Jejunal T cell infiltration was increased in cereal-fed rats compared with HC-fed rats. Also, cereal-fed rats displayed increased *Ifng*/*Tgfb1* and *Ifng*/*Il4* cytokine mRNA ratios, indicating a T_H1-biased immune gene expression profile.

Conclusion: The jejunum of LEW-DP rats exhibited deficits in innate and adaptive immune regulation in association with a pro-inflammatory immune profile compared with LEW-C rats, which were partly overcome by feeding a diabetes-inhibiting low-antigen HC diet.

INTRODUCTION

The traditional rodent models of T1D, NOD mice and BBdp rats, have been invaluable tools for studying pancreatic autoimmunity (33). However, it is increasingly evident that investigation of other animal models is required to gain a better understanding of T1D pathogenesis (38). The LEW-DP strain spontaneously emerged from a colony of MHC congenic LEW.1AR1 rats, with a mutation at the MHC locus resulting in expression of MHC class II *u* molecules (42). Despite this single known mutation upon initial characterization, it was speculated that manifestation of T1D was likely multifactorial and dependent on interactions between multiple genetic risk factors and environmental determinants. Unlike the NOD mouse, there was no sex-bias with respect to T1D incidence, in keeping with BBdp rats and humans (42). Pronounced insulitis was observed in diabetic LEW-DP islets, with infiltrates comprised mainly of T cells, macrophages, and B cells (42), similar to BBdp rats and NOD mice (185). When isolated T cells from lymph nodes and spleen of diabetic LEW-DP rats were adoptively transferred into young ~30 d LEW-DP rats, T1D incidence was increased (42), consistent with the (auto) immune etiology of diabetes in these animals. Interestingly, the MLN from overtly diabetic LEW-DP rats displayed increased numbers of macrophages and T cells compared with asymptomatic rats (185), indicating an association between gastrointestinal inflammation and T1D. In the present study, the role of small intestinal immune homeostasis was explored in young pre-insulitic LEW-DP rats to determine whether alteration of the gut immune infiltrate reflected T1D development, as observed in BBdp rats (see *Chapter 3* and (103)).

RESEARCH OVERVIEW

Rationale

Cereal-based diets promote T1D in two established rodent models, NOD mice and BBdp rats (32; 103; 133). Alterations in small intestinal immune homeostasis have been reported in BBdp rats and NOD mice and could also be implicated in T1D pathogenesis in LEW-DP rats. In *Chapter 3* (103), immune impairments in the jejunum of cereal-fed BBdp rats involving increased T cell infiltration and pro-inflammatory gene expression were observed in association with decreased regulatory capacity. Thus, the LEW-DP jejunum was similarly evaluated to investigate whether gut inflammation was present and modifiable by diet.

Hypotheses

(i) Deficits in gut immune regulation and enhancement of small intestinal inflammation preceding insulitis and T1D are present in the small intestine of LEW-DP rats compared with LEW-C rats. (ii) In LEW-DP rats, diet-associated inhibition of T1D by HC feeding is associated with increased gut immune regulation and decreased inflammation.

Research Questions

1. *Is small intestinal immune homeostasis impaired in LEW-DP rats compared with control LEW-C rats?*

2. *Does weaning onto diabetes-modifying diets, either T1D-promoting cereal or protective HC, alter small intestinal inflammatory status in LEW-DP rats in a manner that reflects dietary modulation of T1D incidence?*

RESULTS

Effect of diet on T1D incidence in LEW-DP rats

At 23 d, LEW-DP rats were fed either a standard cereal diet or a low-antigen HC diet; T1D incidence was determined. LEW-DP rats had a significantly higher T1D incidence when fed a cereal diet compared with an HC diet (**Fig. 4.1**), consistent with BBdp rats and NOD mice (103; 132; 133).

Effect of strain and diet on the distribution of anti-inflammatory CD163$^+$ M2 macrophages

CD163$^+$ cells in LEW rats were distributed in the lamina propria with a secondary subset associated with the epithelium (**Fig. 4.2A**). The number of CD163$^+$ cells in the lamina propria was significantly decreased in LEW-DP rats compared with LEW-C rats (**Fig. 4.2B**). Diet did not modify the number of CD163$^+$ cells among pre-insulitic LEW-DP rats. There were no strain- or diet-associated alterations in the number of epithelium-associated CD163$^+$ cells (**Fig. 4.2C**).

Effect of strain and diet on the distribution of CD3$^+$ T cells

CD3$^+$ cells were distributed within the lamina propria (LPL) with a subset associated with the epithelium (IEL) (**Fig. 4.3A**). The number of CD3$^+$ IEL was significantly decreased in LEW-DP rats compared with LEW-C rats (**Fig. 4.3B**). Among pre-insulitic LEW-DP rats, the number of CD3$^+$ IEL was significantly decreased in HC-fed rats compared with cereal-fed rats. Consistent with CD3$^+$ IEL data, CD3$^+$ LPL were significantly more frequent in LEW-C compared with LEW-DP rats; among LEW-DP rats, there were fewer CD3$^+$ LPL in HC-fed animals compared with cereal-fed counterparts (**Fig. 4.3C**).

Effect of strain and diet on the distribution of CD8α$^+$ T cells

Jejunal T cells were further evaluated by analyzing the subset expressing CD8α. As observed for CD3, CD8α$^+$ cells were distributed within the lamina propria and epithelium (**Fig. 4.3D**). The number of CD8α$^+$ IEL was significantly decreased in LEW-DP rats compared with LEW-C rats (**Fig. 4.3E**). Among pre-insulitic LEW-DP rats, the number of CD8α$^+$ IEL was significantly decreased in HC-fed rats compared with cereal-fed. The number of CD8α$^+$ LPL was significantly larger in LEW-C compared with LEW-DP rats (**Fig. 4.3F**). No significant diet-associated change in CD8α$^+$ LPL was observed among LEW-DP rats

Effect of strain and diet on the distribution of T_{reg} and T_{reg}-associated gene expression

T_{reg} were analyzed by evaluating the Foxp3 transcription factor. Foxp3$^+$ cells were localized exclusively within the lamina propria (**Fig. 4.4A**). When quantified on the basis of mucosal area, the number of Foxp3$^+$ cells was significantly smaller in LEW-DP rats compared with LEW-C rats (**Fig. 4.4B**); no dietary change was observed among pre-insulitic LEW-DP rats. When normalized to the number of CD3$^+$ LPL, the Foxp3$^+$/CD3$^+$ T_{reg} ratio was significantly smaller in LEW-DP rats compared with LEW-C rats (**Fig. 4.4C**). Among pre-insulitic LEW-DP rats, feeding an HC diet significantly increased the Foxp3$^+$/CD3$^+$ T_{reg} ratio compared with cereal feeding. *Foxp3* expression was significantly larger in LEW-C rats compared with LEW-DP rats (**Fig. 4.4D**). Similar to *Foxp3* expression, there was a trend of increased *Ctla4* transcription in LEW-C rats compared with LEW-DP rats (**Fig. 4.4E**). There was a significant positive correlation between expression of *Foxp3* and *Ctla4* (data not shown).

FIGURE. 4.1

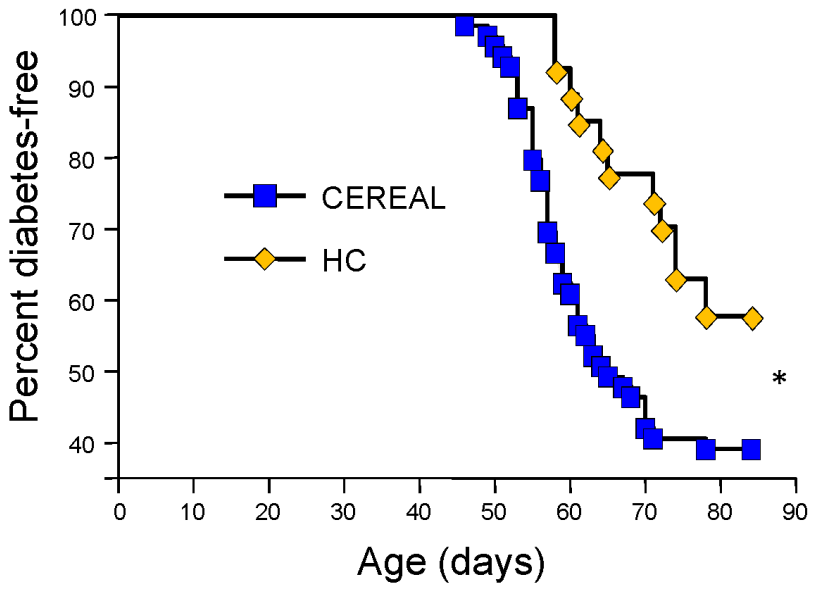

Percent diabetes-free
100
90
80
70
60
50
40
0
10
20
30
40
50
60
70
80
90
Age (days)
CEREAL
HC
*

Figure 4.1. Effect of diet on T1D in LEW-DP rats
Animals were housed under standard SPF conditions and weaned onto a standard cereal-based diet (blue squares) or low-antigen HC diet (yellow diamonds); T1D incidence was determined. Kaplan-Meier survival diagram indicating the percentage of LEW-DP rats that remained free of T1D until ~85 d. P=0.02 as determined by the log-rank test.

FIGURE. 4.2

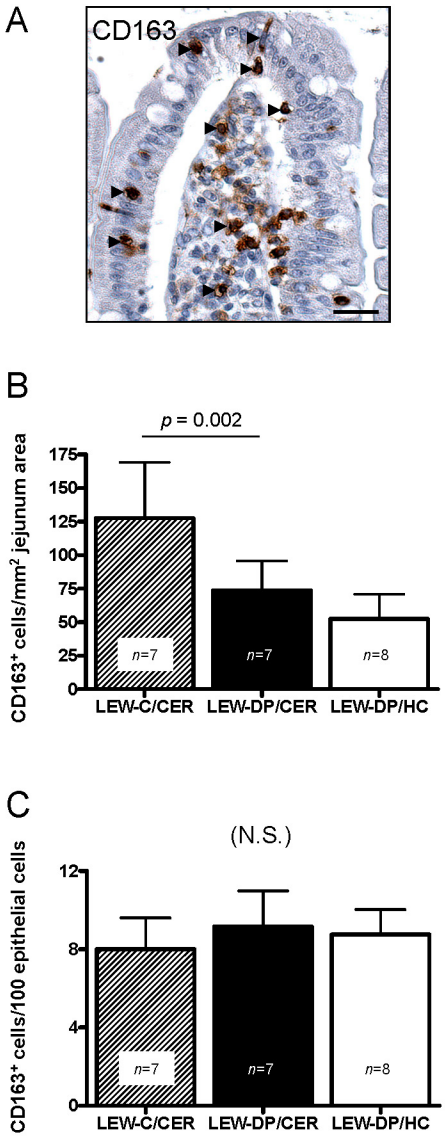

A
CD163
B
p = 0.002
CD163+ cells/mm2 jejunum area
175
150
125
100
75
50
25
0
n=7
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
C
(N.S.)
CD163+ cells/100 epithelial cells
12
8
4
0
n=7
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC

Figure 4.2. Effect of strain and diet on distribution of M2 macrophages
M2 macrophages were evaluated in 45 d cereal-fed LEW-C rats (hatched bars), cereal-fed LEW-DP rats (filled bars), and HC-fed LEW-DP rats (open bars). (**A**) Representative image displaying CD163 staining in jejunum of cereal-fed LEW-DP rat; bar=25 μm. Arrows indicate labeled cells. Quantification of $CD163^+$ cells in (**B**) lamina propria and (**C**) epithelium. *n*=7-8 rats/group. Data represent mean ± SD. *P*-value obtained using ANOVA with LSD post-hoc test.

FIGURE. 4.3

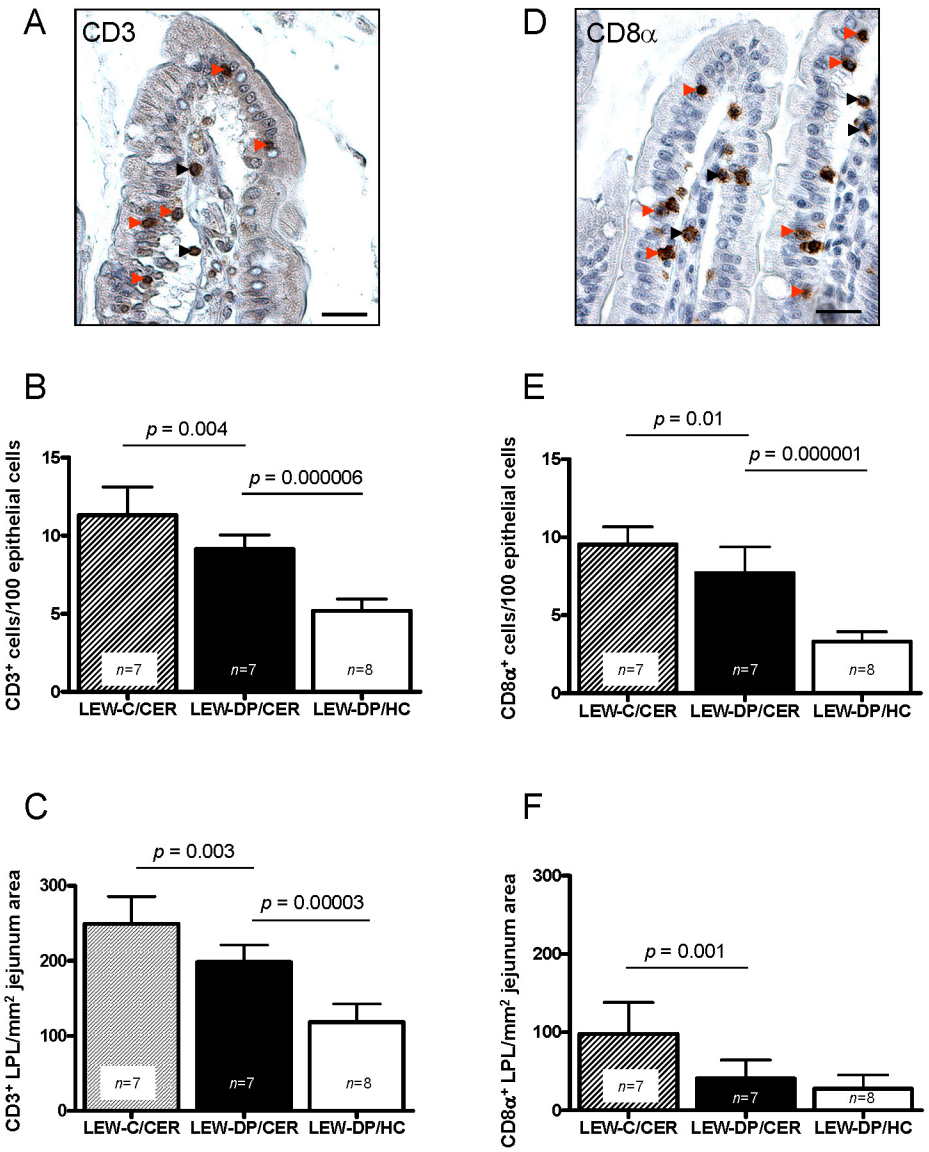

A
CD3
D
CD8α
B
p = 0.004
p = 0.000006
CD3+ cells/100 epithelial cells
15
10
5
0
n=7
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
E
p = 0.01
p = 0.000001
CD8α+ cells/100 epithelial cells
C
p = 0.003
p = 0.00003
CD3+ LPL/mm2 jejunum area
300
200
100
0
F
p = 0.001
CD8α+ LPL/mm2 jejunum area

Figure 4.3. Effect of strain and diet on distribution of T cells

Infiltration of jejunal $CD3^+$ and $CD8\alpha^+$ T cells was evaluated in 45 d cereal-fed LEW-C rats (hatched bars), cereal-fed LEW-DP rats (filled bars), and HC-fed LEW-DP rats (open bars). (**A**) Representative image displaying CD3 staining in jejunum of cereal-fed LEW-DP rat; bar=25 μm. Arrows indicate labeled cells. $CD3^+$ intra-epithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) are indicated by orange and black arrows, respectively. Quantification of $CD3^+$ (**B**) IEL and (**C**) LPL. (**D**) Representative image displaying CD8α staining in jejunum of cereal-fed LEW-DP rat; bar=25 μm. $CD8\alpha^+$ IEL and LPL are indicated by orange and black arrows, respectively. Quantification of $CD8\alpha^+$ (**E**) IEL and (**F**) LPL. *n*=7-8 rats/group. Data represent mean ± SD. *P*-values obtained using ANOVA with LSD post-hoc test.

FIGURE. 4.4

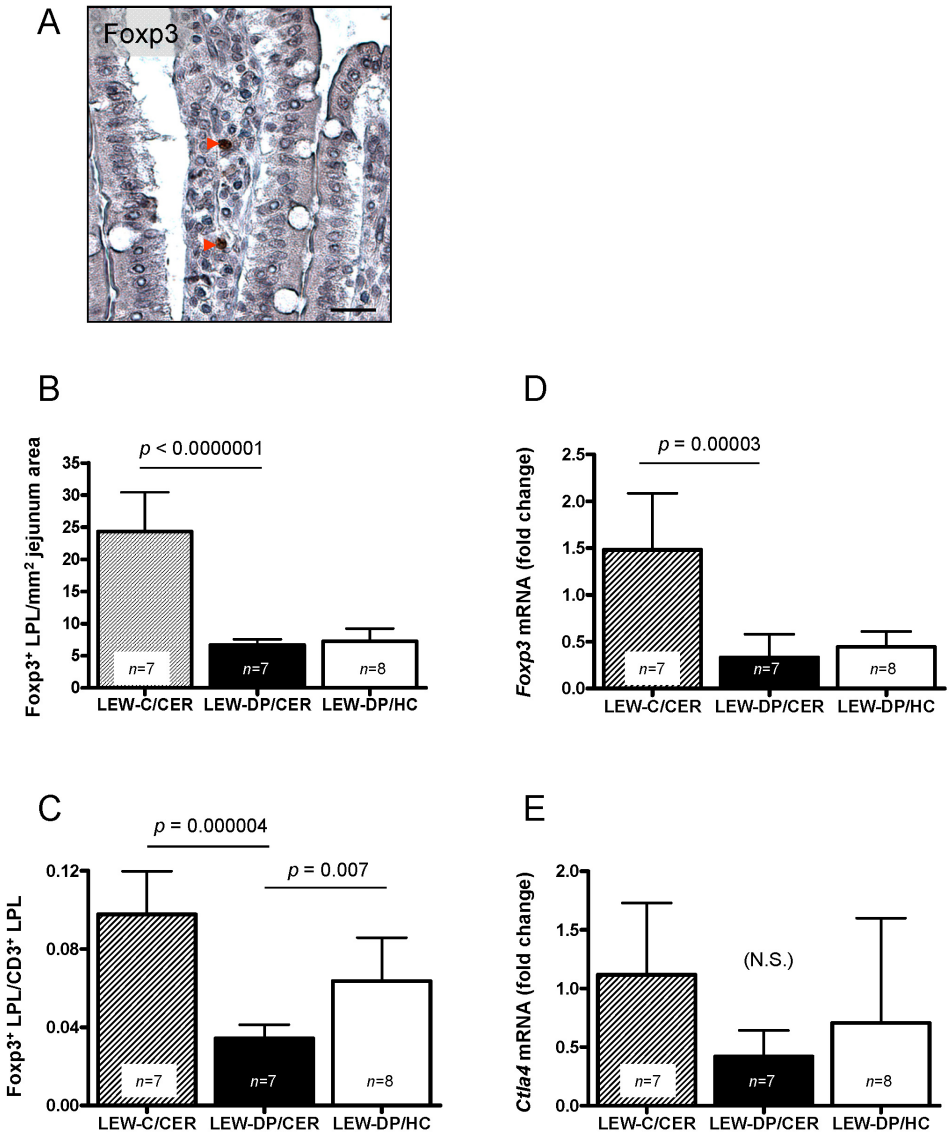

A
Foxp3
B
Foxp3+ LPL/mm2 jejunum area
p < 0.0000001
n=7
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
D
Foxp3 mRNA (fold change)
p = 0.00003
C
Foxp3+ LPL/CD3+ LPL
p = 0.000004
p = 0.007
E
Ctla4 mRNA (fold change)
(N.S.)

Figure 4.4. Effect of strain and diet on regulatory T cells
Infiltration of jejunal $Foxp3^+$ T cells was evaluated in 45 d cereal-fed LEW-C rats (hatched bars), cereal-fed LEW-DP rats (filled bars), and HC-fed LEW-DP rats (open bars). (**A**) Representative image displaying Foxp3 staining in jejunum of cereal-fed LEW-DP rat; bar=25 μm. $Foxp3^+$ lamina propria lymphocytes (LPL) are indicated by red arrows. (**B**) Number of $Foxp3^+$ LPL/mm^2 jejunum area. (**C**) Ratio of $Foxp3^+$ LPL/$CD3^+$ LPL. *n*=7-8 rats/group. Data represent mean ± SD. *P*-values obtained using ANOVA and by LSD post-hoc test. Transcription of T_{reg}-enriched genes in jejunum of 45 d LEW rats. Gene expression of (**D**) *Foxp3* and (**E**) *Ctla4*. *n*=7-8/group. Data represent mean ± SD. *P*-values obtained using ANOVA followed by LSD post-hoc test.

Associations between Foxp3$^+$ T_{reg} and T cells

T cell homeostasis was evaluated by comparing the distribution of total T cells and T_{reg}. Significant negative correlations were observed between the numbers of CD3$^+$ IEL and the Foxp3$^+$/CD3$^+$ T_{reg} ratio (**Fig. 4.5A**); CD3$^+$ LPL and Foxp3$^+$/CD3$^+$ (**Fig. 4.5B**); CD8α^+ IEL and Foxp3$^+$/CD3$^+$ (**Fig. 4.5C**); no correlation was observed between CD8α^+ LPL and Foxp3$^+$/CD3$^+$ (**Fig. 4.5D**). Thus, increasing T_{reg} proportion was associated with decreasing total T cell infiltration among LEW-DP rats. Maturation and shaping of the immune phenotype in the small intestine could be altered over time. Jejunum samples from 45 d and 100 d rats were compared to evaluate age-related modification of T_{reg} distribution (**Fig. 4.5E**). In LEW-C rats, no significant age-associated alteration in the Foxp3$^+$/CD3$^+$ T_{reg} ratio was observed. However, the Foxp3$^+$/CD3$^+$ T_{reg} ratio was significantly smaller in 100 d cereal-fed LEW-DP compared with 45 d rats. The Foxp3$^+$/CD3$^+$ ratio was not significantly modified by age in HC-fed LEW-DP rats. Thus, T cell homeostasis was impaired with increasing age only in cereal-fed LEW-DP rats whereas diabetes-resistant LEW-C rats and diet-protected LEW-DP rats maintained stable T_{reg} balance.

Effect of strain and diet on cytokine gene expression

Expression of genes encoding various cytokines was evaluated to determine the effects of strain and diet in LEW rats. There were no significant strain- or diet-associated differences in expression of either *Ifng* (**Fig. 4.6A**) or *Il15* (**Fig. 4.6B**). A significant positive correlation was observed between expression profiles of both genes (data not shown). No significant changes in expression of genes encoding the T_H2 and T_H17-associated cytokines *Il4* (**Fig.**

4.6C) and *Il17a* (**Fig. 4.6D**) were observed. Lastly, no significant differences in expression of T_{reg}-associated genes *Il10* (**Fig. 4.6E**) or *Tgfb1* (**Fig. 4.6F**) were observed.

Effect of strain and diet on jejunal T_H transcriptional bias

Small intestinal mRNA expression ratios of major hallmark cytokines were evaluated as a reflection of T_H polarization bias in LEW rats. There was no significant strain-associated difference in the *Ifng*/*Il4* expression ratio, reflective of T_H1/T_H2 bias, between LEW-C and LEW-DP rats (**Fig. 4.7A**). However, among LEW-DP rats, the *Ifng*/*Il4* was significantly larger in cereal-fed rats compared with HC-fed. The *Ifng*/*Tgfb1* ratio, reflective of T_H1/T_{reg} expression bias, was significantly increased in LEW-DP rats compared with LEW-C rats (**Fig. 4.7B**). Also, there was a trend of a dietary effect among diabetes-prone rats, with feeding of an HC diet resulting in decreased *Ifng*/*Tgfb1* mRNA ratio compared with cereal feeding (p=0.05). There were no significant strain- or diet-associated differences in the ratio of *Ifng*/*Il10*, reflective of T_H1/T_{reg} expression bias (**Fig. 4.7C**). Thus, in cereal-fed LEW-DP rats, T_H1-associated expression of *Ifng* predominated over major immunomodulatory cytokines *Il4* and *Tgfb*.

SUMMARY

In the period preceding T1D, there was a deficit in total T cell numbers in LEW-DP rats compared with LEW-C rats. In addition, LEW-DP rats displayed deficits in anti-inflammatory immune cell subsets compared with resistant LEW-C rats. Among LEW-DP animals, HC feeding decreased the number of infiltrating T cells, resulting in an increased tolerogenic $Foxp3^+/CD3^+$ T_{reg} ratio. Also, HC feeding decreased the pro-inflammatory T_H1-biased transcriptional profile compared with cereal feeding among LEW-DP rats.

FIGURE. 4.5

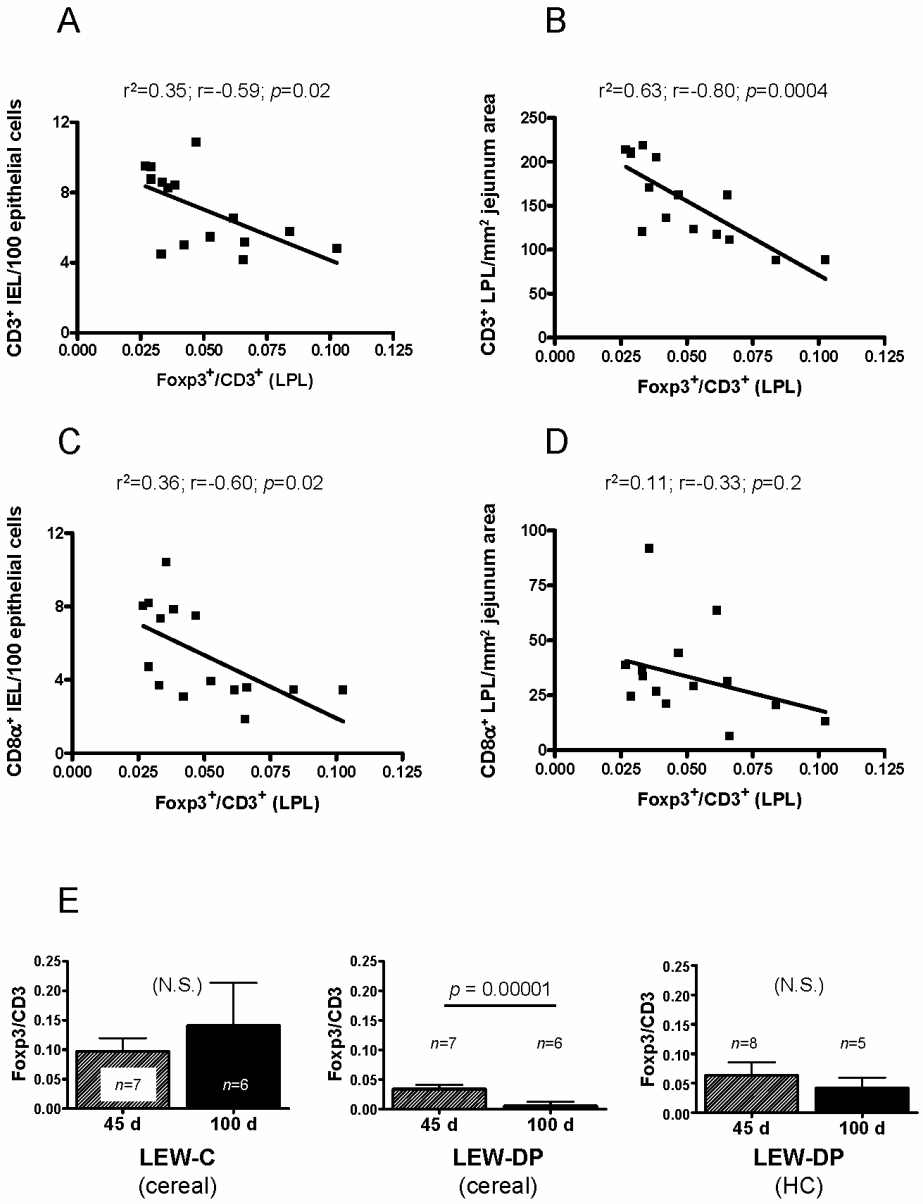

A
r²=0.35; r=-0.59; p=0.02
CD3⁺ IEL/100 epithelial cells
Foxp3⁺/CD3⁺ (LPL)
B
r²=0.63; r=-0.80; p=0.0004
CD3⁺ LPL/mm² jejunum area
Foxp3⁺/CD3⁺ (LPL)
C
r²=0.36; r=-0.60; p=0.02
CD8α⁺ IEL/100 epithelial cells
Foxp3⁺/CD3⁺ (LPL)
D
r²=0.11; r=-0.33; p=0.2
CD8α⁺ LPL/mm² jejunum area
Foxp3⁺/CD3⁺ (LPL)
E
Foxp3/CD3
(N.S.)
n=7
n=6
45 d
100 d
LEW-C
(cereal)
p = 0.00001
n=7
n=6
LEW-DP
(cereal)
(N.S.)
n=8
n=5
LEW-DP
(HC)

Figure 4.5. Associations between Foxp3$^+$ T_{reg} and T cells in LEW-DP rats and effect of age on T cell-T_{reg} homeostasis at 45 d and 100 d

The association between the proportion of Foxp3$^+$ T_{reg} and T cell subsets in the jejunum of LEW-DP rats was evaluated in 45 d animals. Negative correlations between the numbers of (**A**) CD3$^+$ IEL and the Foxp3$^+$/CD3$^+$ ratio; (**B**) CD3$^+$ LPL and Foxp3$^+$/CD3$^+$; (**C**) CD8α^+ IEL and Foxp3$^+$/CD3$^+$; (**D**) no significant correlation between CD8α^+ LPL and Foxp3$^+$/CD3$^+$. *n*=15. Correlation coefficients obtained using Pearson's correlation; *p*-values obtained by linear regression analysis. Effect of age on T cell homeostasis in LEW-C and LEW-DP rats. (**E**) Foxp3/CD3 T_{reg} ratios in cereal-fed LEW-C rats, cereal-fed LEW-DP, and HC-fed LEW-DP rats. *n*=5-8 rats/group. Data represent mean ± SD. *P*-values obtained using Student's *t*-test.

FIGURE. 4.6

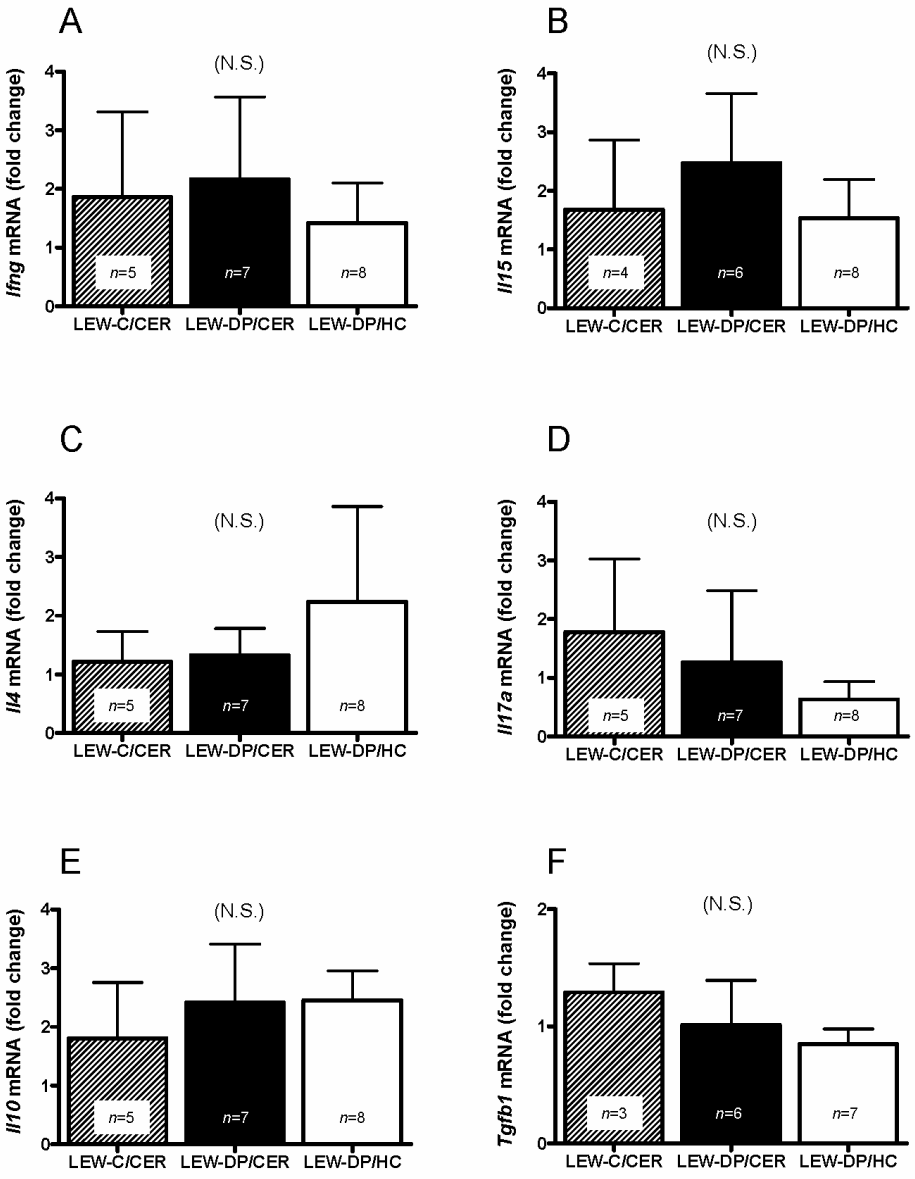

A
(N.S.)
Ifng mRNA (fold change)
n=5
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
B
(N.S.)
Il15 mRNA (fold change)
n=4
n=6
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
C
(N.S.)
Il4 mRNA (fold change)
n=5
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
D
(N.S.)
Il17a mRNA (fold change)
n=5
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
E
(N.S.)
Il10 mRNA (fold change)
n=5
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
F
(N.S.)
Tgfb1 mRNA (fold change)
n=3
n=6
n=7
LEW-C/CER
LEW-DP/CER
LEW-DP/HC

Figure 4.6. Effect of strain and diet on jejunal expression of cytokine genes
Small intestinal mRNA expression of cytokines genes was evaluated in the jejunum of 45 d cereal-fed LEW-C rats (hatched bars), cereal-fed LEW-DP rats (filled bars), and HC-fed LEW-DP rats (open bars). Gene expression of (**A**) *Ifng*, (**B**) *Il15*, (**C**) *Il4*, (**D**) *Il17a*, (**E**) *Il10*, and (**F**) *Tgfb1*. *n*=3-8/group. Data represent mean ± SD. Statistics performed using ANOVA followed by LSD post-hoc test.

FIGURE. 4.7

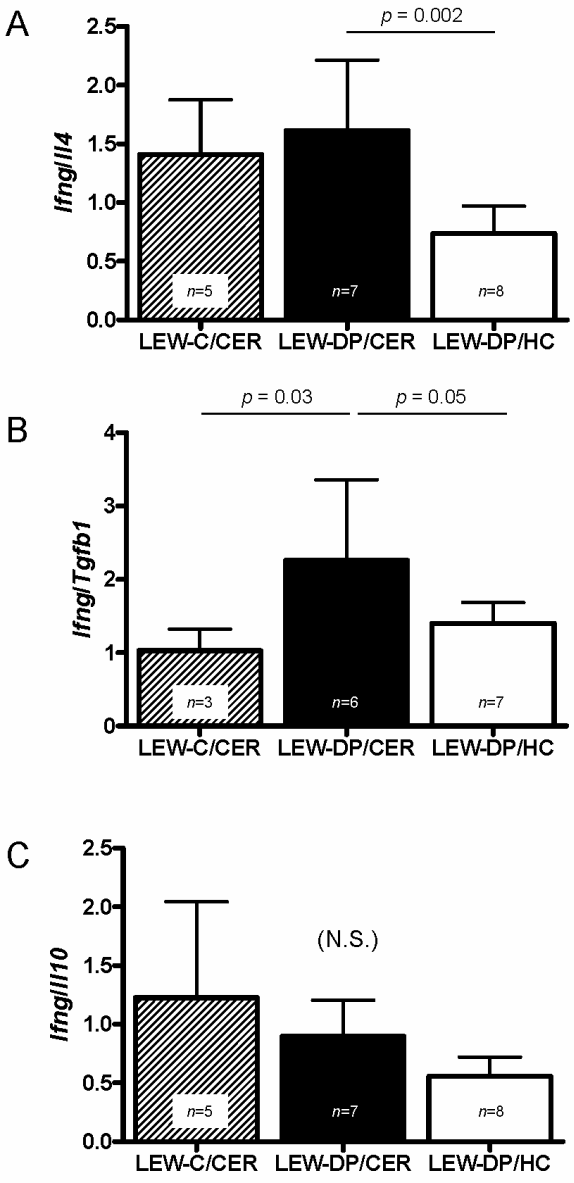

A
p = 0.002
Ifng/Il4
2.5
2.0
1.5
1.0
0.5
0.0
n=5
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
B
p = 0.03
p = 0.05
Ifng/Tgfb1
4
3
2
1
0
n=3
n=6
n=7
LEW-C/CER
LEW-DP/CER
LEW-DP/HC
C
Ifng/Il10
2.5
2.0
1.5
1.0
0.5
0.0
(N.S.)
n=5
n=7
n=8
LEW-C/CER
LEW-DP/CER
LEW-DP/HC

Figure 4.7. Effect of strain and diet on jejunal T_H transcriptional bias
Small intestinal mRNA expression ratios of major hallmark cytokines as a reflection of T_H transcriptional bias, were determined in the jejunum of 45 d cereal-fed LEW-C rats (hatched bars), cereal-fed LEW-DP rats (filled bars), and HC-fed LEW-DP rats (open bars). Gene expression of (**A**) *Ifng*/*Il4*, (**B**) *Ifng*/*Tgfb1*, and (**C**) *Ifng*/*Il10*. *n*=3-8/group. Data represent mean ± SD. *P*-values obtained using ANOVA followed by LSD post-hoc test.

Thus, the jejunum of LEW-DP rats displayed mildly reduced T cell numbers and decreased immune regulatory potential compared with LEW-C rats, some aspects of which were reversible by weaning onto a diabetes-protective HC diet. Major changes in intestinal immune homeostasis that were observed LEW rats are represented in a simplified diagram (**Fig. 4.8**).

DISCUSSION

The role of the gut immune system and dietary antigens was explored for the first time in LEW-DP rats, an alternative rodent model of spontaneous T1D. Immune cell infiltrates in the jejunum of LEW-DP rats were compared with infiltrates from diabetes-resistant LEW-C rats to establish whether modification of the small intestinal immune phenotype was a feature of the diabetes-prone strain. Pre-insulitic 45 d LEW-DP rats displayed significantly fewer T cells in the epithelium and lamina propria compared with LEW-C rats. These findings are indicative of mild intestinal T cell deficit in the period preceding T1D onset. A recent report described T cell abnormalities in LEW-DP rats compared with LEW-C counterparts (186). Arndt *et al*. reported the occurrence of a variable $CD3^+$ T cell frequency in the circulation of LEW-DP rats compared with control rats, a condition that was associated with T1D development (186). In animals aged 35-110 d, the variability in the percentage of $CD3^+$ T cells in asymptomatic and overtly diabetic LEW-DP rats was larger compared with LEW-C animals (186). The mean percentage of $CD3^+$ T cells in peripheral blood was also decreased in asymptomatic LEW-DP rats compared with LEW-C rats (186). Furthermore, the numbers of both $CD3^+$ and $CD8^+$ T cells were decreased in overtly diabetic LEW-DP rats compared with asymptomatic LEW-C rats (186).

FIGURE. 4.8

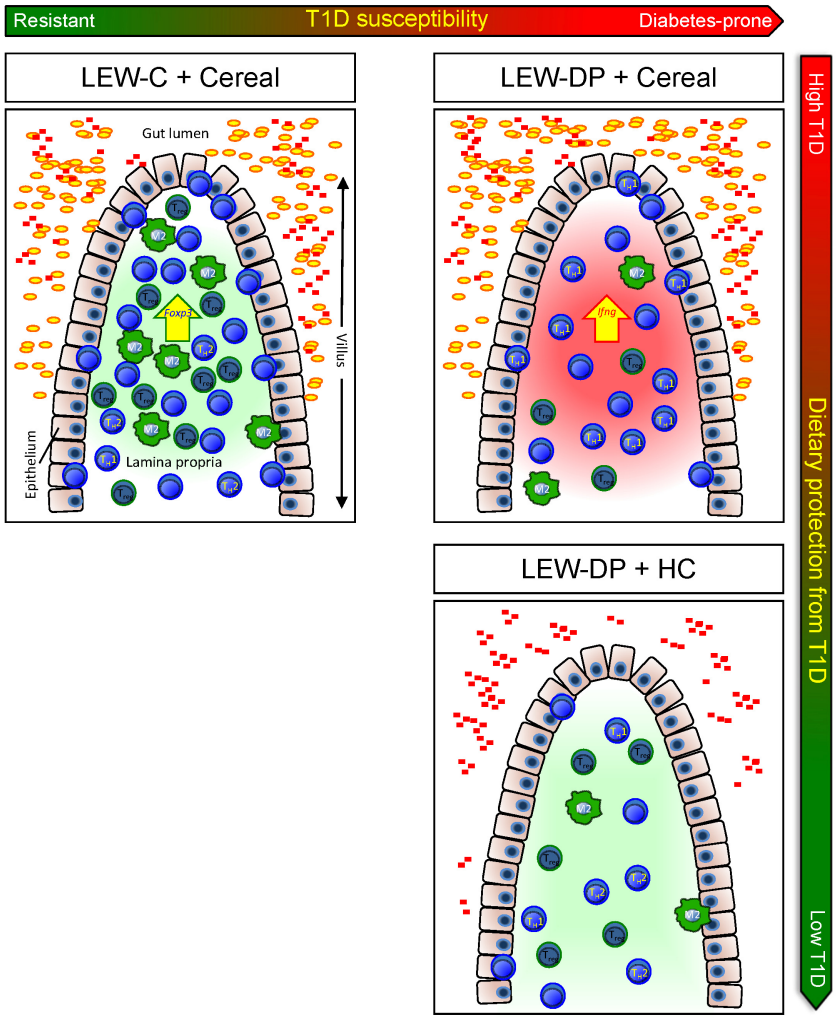

Resistant
T1D susceptibility
Diabetes-prone
LEW-C + Cereal
LEW-DP + Cereal
LEW-DP + HC
Gut lumen
Villus
Epithelium
Lamina propria
foxp3
ifng
High T1D
Dietary protection from T1D
Low T1D

Figure 4.8. Overview of gut immune profile in LEW-DP rats
Simplified diagram (non-scaled) displaying major alterations of immune cell distribution and immunological gene expression in the jejunum of young LEW-C and LEW-DP rats. Strain-related differences associated with T1D susceptibility (LEW-C vs. LEW-DP) and dietary differences (cereal vs. HC) associated with altered T1D incidence are emphasized. LEW-C rats, cereal-fed (upper left panel), LEW-DP rats, cereal-fed (upper right panel), and LEW-DP, HC-fed (lower right panel). Upper panels (left to right) indicate the strain-related small intestinal modification of immune phenotype observed upon comparison of 45 d (cereal-fed) LEW-C rats and LEW-DP rats. Right-side panels (top to bottom) indicate diet-related small intestinal modification of immune phenotype in cereal-fed LEW-DP rats compared with HC-fed LEW-DP rats. Strain- and diet-associated T1D risk among LEW rats is characterized by a combination of deficits in anti-inflammatory immune cell populations in parallel with increased T cell infiltrates in the context of a T_H1-biased transcriptional microenvironment. Character legend: *Antigens* - Yellow ovals: cereal proteins; red rectangles: microbes. *Immune cells* - Blue: effector T cells; green: $CD163^+$ M2 macrophages; teal: $Foxp3^+$ T_{reg}.

In the present study, decreased T cell numbers were similarly observed in the jejunum of LEW-DP rats compared with LEW-C rats, consistent with the Arndt *et al*. study. Taken together, these findings indicate that mild T cell deficits, whether in the periphery or small intestine, represent an important immune feature of the LEW-DP strain, suggesting dysregulated gut immune homeostasis is associated with T1D susceptibility. Arndt *et al*. speculated that T cell variability and T cell deficit could be linked to an imbalance in effector T cells and T_{reg} in LEW-DP rats, resulting in chronic inflammation and potentiation of T1D (186). The T cell deficit and associated imbalance between total T cells and T_{regs} contributes to T1D in BBdp rats (38; 187) and could also be implicated in pathogenesis in LEW-DP rats.

Interestingly, other findings described by Wedekind *et al*. are consistent with a regulatory deficit and lymphopenic state representing important drivers of T1D in the LEW-DP strain (188). Adoptive transfer of T cells from overtly diabetic LEW-DP rats into recipient LEW-C rats did not induce T1D, whereas transplant of these cells into LEW-DP rats resulted in a two-fold increase in incidence (188). Thus, enhanced baseline immunoregulatory capacity in the LEW-C strain relative to LEW-DP rats was sufficient to inhibit the adoptively transferred diabetogenic cells from precipitating T1D in the resistant control strain (188). Conversely, when T cells from LEW-C rats were adoptively transferred into LEW-DP recipients, T1D incidence was significantly smaller, demonstrating that correction of the T cell deficit and immune regulation afforded significant protection from disease (188). The T cell deficit observed in the jejunum of LEW-DP rats was accompanied by alterations in T_{reg} homeostasis, consistent with Wedekind *et al*. (188). LEW-DP rats displayed a smaller proportion of small intestinal $Foxp3^{+}$ T_{reg} compared with diabetes-resistant LEW-C rats, which was ameliorated by feeding of a diabetes-inhibiting HC diet.

Also, LEW-DP rats displayed decreased *Foxp3* gene expression compared with LEW-C rats. Badami *et al*. reported a deficit in gut $Foxp3^+$ T_{reg} in duodenal biopsies of patients with T1D compared with non-diabetic controls (189), indicating that regulatory impairment in the small intestine also characterizes some patients with T1D. Interestingly, the deficit was linked to the inability of lamina propria DC from T1D patients to promote differentiation of $Foxp3^+$ T_{reg} (189), indicating impairment of innate immunoregulation in the small intestine.

The tolerogenic function of T_{reg} is imparted by a variety of factors (94) including production of TGF-β, IL-10, and CTLA-4 (190; 191). Instability of the T_{reg} phenotype, characterized in part by decreased Foxp3, could lead to reduction of tolerogenic function and even adoption of pro-inflammatory effector function (192-194). TGF-β1 is critical for maintenance of T_{reg} and contributes to suppression of pro-inflammatory immune responses by inhibiting the activation of effector T cells (195). In parallel with a deficit in $Foxp3^+$ T_{reg} and *Foxp3* mRNA, young LEW-DP rats also displayed an increased *Ifng*/*Tgfb1* mRNA ratio compared with LEW-C rats. These abnormalities were partly corrected in LEW-DP rats by weaning the animals onto a diabetes-protective HC diet, which increased the proportion of $Foxp3^+$ T_{reg} and decreased T_H1-biased transcription. Interestingly, weaning onto a low-antigen diet also stabilized the proportion of $Foxp3^+$ T_{reg} during the period encompassing T1D development, with similar $Foxp3^+/CD3^+$ T_{reg} ratios observed at 45 d and 100 d in HC-fed LEW-DP rats. Similarly in cereal-fed LEW-C rats, a stable $Foxp3^+/CD3^+$ T_{reg} ratio was observed at 45 d and 100 d. In contrast, $Foxp3^+$ T_{reg} proportion in cereal-fed LEW-DP rats, which was already decreased relative to HC-fed LEW-DP counterparts, decreased further at 100 d. These results indicate that the small intestinal T_{reg} decline was progressive in LEW-

DP rats fed the diabetes-promoting cereal-based diet. Thus, weaning LEW-DP rats onto an HC diet enabled establishment of a stable gut immune phenotype resembling the tolerogenic, anti-inflammatory profile of the LEW-C jejunum.

The dramatic consequences of *Foxp3* disruption and T_{reg} imbalance underscore the critical importance of immune regulation in protecting against inflammation and autoimmunity (94). Mice with a deletion or loss-of-function mutation in the *Foxp3* gene (*Scurfy* mice) develop a fatal autoimmune phenotype characterized by pronounced multi-organ inflammation (94). Mutation of the human *FOXP3* gene causes the monogenic IPEX syndrome, features of which are consistent with the phenotype of *Scurfy* mice, with autoimmune diabetes and hyperglycemia developing in the majority of patients, even in the neonatal period (96; 196). In parallel with islet inflammation, IPEX is also characterized by dramatic intestinal infiltration by immune cells, suggesting potentially common pathways of intestinal and pancreatic inflammation in the absence of appropriate immune regulation even in monogenic forms of diabetes (96). For example, a decreased proportion of $Foxp3^+$ T_{reg} was reported in MLN cells from 16 week-old BB-DP rats compared with BB-DR rats (187). Also, adoptive transfer of T_{reg} inhibited T1D in BB-DP rats whereas depletion of these cells induced T1D in BB-DR rats (38). Thus, T_{reg} can inhibit T1D and a deficit in these cells in the jejunum of LEW-DP rats is consistent with increased susceptibility. Despite likely playing a permissive role in the pathogenesis of celiac disease, there is scarce information on the effects of cereal exposure on T_{reg} homeostasis. Compared with a gluten-free diet, feeding wheat gluten resulted in fewer intestinal Peyer's patch-associated T_{reg} in BALB/c mice (197). Deficits or impairments in $Foxp3^+$ T_{reg} could stimulate or exacerbate aberrant pro-inflammatory immune responses upon exposure to cereal proteins.

In addition to a T_{reg} deficit, young LEW-DP rats also displayed a significant decrease in the number of anti-inflammatory $CD163^+$ M2 macrophages compared with resistant LEW-C rats. Previous data from our group revealed a deficit in $CD163^+$ M2 macrophages in the jejunum of BBdp rats compared with resistant BBc rats (103), in keeping with the importance of $CD163^+$ M2 macrophages providing resistance to spontaneous T1D in rat models. Furthermore, adoptive transfer of M2 macrophages inhibited T1D in NOD mice, clearly demonstrating the potent regulatory capacity of this innate immune population (102). In humans, the immunomodulatory cytokine IL-10 induces *CD163* transcription whereas pro-inflammatory mediators IFN-γ, TNF-α, and LPS are inhibitory (198). Thus, a regulatory immune microenvironment favours development of tolerogenic macrophages. T_{reg} directly support the expansion and function of M2 macrophages. For example, co-culture of monocytes and macrophages with T_{reg} induced expression of M2-associated scavenger receptors CD163 and CD206 in association with decreased MHC class II expression and decreased pro-inflammatory responsiveness to LPS stimulation (199). Thus, a deficit in $Foxp3^+$ T_{reg} and *Foxp3* gene expression in LEW-DP rats compared with LEW-C rats could have directly contributed to impaired expansion and development of M2 macrophages. Conversely, the deficit in M2 macrophages could have prevented differentiation and expansion of T_{reg} in the LEW-DP jejunum. M2 macrophages directly promote the induction of $Foxp3^+$ T_{reg} via a mechanism dependent on exposure to a combination of secreted factors, including TGF-β, IL-10, and retinoic acid (105). In the present study, the *Ifng*/*Tgfb1* mRNA ratio was increased in young LEW-DP rats compared with LEW-C rats, consistent with a mechanism of T_H1 cytokine-associated inhibition of T_{reg} development, potentially linked in part to an underlying deficit in $CD163^+$ M2 macrophages. Thus, M2 macrophages are

integrated into immunological networks directly implicated in the establishment of $Foxp3^+$ T_{reg} (105; 106). The complementary interplay between M2 macrophages and T_{reg} is additive and critical for prevention of chronic inflammation. Both cell types were deficient in the jejunum of LEW-DP rats, likely contributing to sensitivity to provocative environmental antigens (e.g. cereal proteins) and conferring T1D risk. The frequency of human $FOXP3^+$ cells in peripheral blood of patients with T1D was not different compared with control subjects and did not change according to disease stage (200). However, as suggested by studies of celiac disease and the current findings, the gut represents a more informative organ for quantification of anti-inflammatory cells, especially when evaluating regulatory capacity towards normally innocuous environmental factors.

Consistent with findings from BBdp rats, dietary promotion of T1D in LEW-DP rats was also associated with increased intestinal T cell infiltration and T_H1-biased transcription compared with HC-fed rats. Increased transcription of *IFNG* was also observed in biopsies from children with untreated celiac disease compared with control children and feeding a gluten-free diet for one year decreased *IFNG* expression (201), indicating a causal role for cereal antigens in T_H1 inflammation. Interestingly, these alterations were not detected in blood (201), again suggesting that mucosal inflammation is not necessarily reflected at extra-intestinal sites and should also be considered *in situ*, as done in the present study. Cereal feeding in LEW-DP rats increased T cell infiltration in association with development of a T_H1-biased transcriptional profile compared with protected HC-fed LEW-DP counterparts. Thus, the effect of cereal exposure on the small intestinal immune phenotype could be similar in subjects susceptible to celiac disease and T1D. Using an NOD model of non-spontaneous T1D (NOD-DQ8 mice), Galipeau *et al*. reported that gliadin sensitization

stimulated IEL infiltration (202). The gliadin sensitization protocol involved administration by gavage of a pepsin-trypsin digest of gliadin with cholera toxin followed by a switch to a gluten-containing diet; controls were administered cholera toxin but not subsequently fed a gluten-containing diet (202). Interestingly, pronounced insulitis was inducible in this model when gliadin sensitization was done in parallel with partial depletion of $Foxp3^+$ T_{reg} using anti-CD25 antibodies (202). Thus, when an immune sensitivity to cereal antigens is present in a diabetes-prone genetic setting, a deficit in immune regulation is sufficient for induction of insulitis, whereas appropriate regulation can restrict the inflammation to the gut (202). A deficit in regulatory potential characterized the small intestinal immune microenvironment of LEW-DP rats relative to the control LEW-C strain. In the context of deficient immunoregulation, exposure to cereal proteins potentially stimulated gut T cell infiltration and promoted T1D in LEW-DP rats. Taken together, these data suggest a common pathway of cereal-associated gut inflammation and T1D promotion in diabetes-prone rodent models.

CONCLUSION

T1D susceptibility in LEW-DP rats was associated with an inflammatory phenotype in the jejunum, characterized by a deficit in T cells and immune regulation. Also, environmental modification of T1D incidence by diet in LEW-DP rats was accompanied by immune-associated alterations in the jejunum. HC-associated protection was associated with a shift in gut immune profile towards an anti-inflammatory phenotype in conjunction with decreased T cell infiltration and decrease in T_H1-biased transcription, similar to observations made in BBdp rats (see *Chapter 3* and (103)).

CHAPTER 5. CAMP host defense peptide as a novel gut-pancreas nexus implicated in modulation of T1D

ABSTRACT

Background: Weaning BBdp rats onto an HC diet resulted in enrichment of anti-inflammatory M2 macrophages and inhibition of T cell infiltration, effects potentially contributing to T1D protection.

Methods: Immune-focused PCR Arrays were used for identification of novel factors differentially regulated by diet in the jejunum of SPF-housed asymptomatic 130 d BBdp rats fed either cereal or HC. Additional characterization of a novel diet-modifiable candidate gene, *Camp* (cathelicidin antimicrobial peptide/CAMP), was performed using RT-qPCR, immunohistochemistry, and confocal microscopy. Also, CAMP/LL-37 injections in BBdp rats were performed to evaluate the potential for modification of microbiota, mucosal immunity, and islet biology in BBdp rats.

Results: Expression of *Camp* was increased in the jejunum of diet-protected HC-fed BBdp rats. Jejunal CAMP co-localized with CD163, indicating an association with anti-inflammatory M2 macrophages. Unexpectedly, CAMP was also observed in PLN and islets, associated with $CD163^{+}$ M2 macrophages and a subset of β-cells, respectively. Islet-associated CAMP correlated with total islet number, indicating an association with islet expansion. CAMP/LL-37 injections increased the abundance of probiotic bacteria and promoted islet neogenesis.

Summary: HC feeding induced jejunal expression of *Camp*, a multifunctional host defense factor. CAMP was also distributed in PLN and islets and represents a novel diabetes-associated factor linking microbes, gut immunity, pancreatic immunity and islet homeostasis.

INTRODUCTION

Studies described in *Chapter 3* (also see (103)) demonstrated that weaning BBdp rats onto a diabetes-inhibiting HC diet was reflected by inhibition of jejunal T cell infiltration and decreased T_H1-biased gene expression in parallel with enrichment of anti-inflammatory $CD163^+$ M2 macrophages. Key objectives of the present study were to expand on potential mechanisms underlying the dietary modification of gut immunity in BBdp rats and to identify potential $CD163^+$ M2 macrophage-associated factors associated with anti-inflammatory function.

Attempts to prevent or reverse T1D have focused mostly on adaptive immunity, including anti-CD3 antibody treatment or non-specific immunosuppression (e.g. cyclosporin). However, these trials have mostly failed, with only temporary or minor benefit (203). A greater understanding of the role of innate immunity in the pathogenesis of T1D is essential for development of novel prevention and treatment strategies. Previously unrecognized associations between innate immunity and T1D pathogenesis are being uncovered. For example, impaired TLR signalling was observed in peripheral monocytes from autoantibody-positive subjects causing increased secretion of IL-1β (204), a major pro-inflammatory cytokine (205). In two recent randomized placebo-controlled clinical trials, IL-1 antagonism was evaluated as a potential therapy for T1D (206). IL-1β or the IL-1 receptor were antagonized in subjects with recent-onset T1D, but the interventions failed to increase C-peptide concentration and preserve β-cell mass (206). Attempting to block individual pro-inflammatory factors is unlikely to successfully reverse T1D at such late stages of the disease process. Targeting innate immunity in novel ways, including supplementation of anti-

inflammatory innate factors could be an effective alternative strategy for treatment. The inhibition of T1D in NOD mice by adoptive transfer of M2 macrophages demonstrates this point (102). A primary goal of the present study was to identify specific factors associated with the anti-inflammatory effect of M2 macrophages that could inhibit T1D. One means of searching for T1D-related candidate molecules is gene expression profiling. Such a strategy was previously used by Hessner *et al*. and revealed novel involvement of the innate immune factor eotaxin-1 in T1D pathogenesis in BB rats (39).

An intriguing aspect of innate immunity that has received little attention in the context of T1D is antimicrobial peptides (AMPs). The role of microbes in T1D is an active area of research (207-209) and yet investigation of the role of AMPs has only just begun (103). Altered AMP status could represent the basis for altered microbiota observed in BB-DP rats compared with BB-DR rats (87). NOD mice deficient in MyD88, a key TLR-associated innate immune signalling adaptor molecule, were protected from T1D under standard SPF housing conditions, demonstrating a critical role for innate immunity in T1D modulation (169). Interestingly, protection was not observed when animals were housed under germ-free conditions, suggesting that a subset of protective commensal bacteria likely contributed to T1D inhibition (169). An interesting question is whether certain AMPs contribute to intestinal homeostasis by promoting enrichment of probiotic bacteria. Probiotic bacteria are associated with protection from gastrointestinal diseases (210) and also inhibited T1D in NOD mice (211; 212). Consistent with this, increased fecal abundance of probiotic bacteria *Lactobacillus* and *Bifidobacterium* was reported in resistant BB-DR rats compared with BB-DP rats (87). Thus, probiotic bacteria contribute to T1D protection in rodent models. There are two major classes of AMPs that possess net positive charges and have an

amphipathic structure: (i) α-helical peptides that lack cysteine residues and disulfide bonds, the main example being cathelicidin antimicrobial peptide (CAMP); and (ii) β-sheet globular peptides such as β-defensins, which contain cysteine residues that form multiple intramolecular disulfide bridges (213). As Gallo and Hooper recently remarked, it is fascinating to consider that many AMPs have retained their "potent functionality over evolutionary timescales" (214), underscoring their importance in mammalian health. CAMP function could involve enrichment of probiotic bacteria associated with beneficial effects on gut homeostasis. This concept has precedence, as BB-DP rats administered the probiotic strain *L. johnsonii* N6.2 had lower T1D in association with decreased intestinal *Ifng* expression (88).

The antimicrobial capacity of CAMP is broad, with anti-bacterial, anti-viral, and anti-fungal activity reported (214; 215). The potential involvement of CAMP in altering microbial composition provides an exciting opportunity to explore the role of this factor in T1D pathogenesis. AMPs collectively display a broad range of homeostatic and repair functions associated with immunomodulation, growth, and wound healing, most notably at environment-exposed sites including skin, respiratory tract, genitourinary tract, and gastrointestinal tract (215; 216). These properties could contribute to the modulation of various chronic inflammatory diseases, including T1D. A key point is that CAMP engages a wide variety of signalling pathways by interacting with a large variety of mammalian (host) receptors, including growth factor receptors, chemokine receptors, and TLRs (217). Thus, CAMP is imbued with the unique capacity to affect both microbes and host in vastly different ways. Given its multifunctional nature, CAMP is potentially involved in shaping the microbiota, modifying immune microenvironments, and mediating tissue growth. The

present *Chapter* (see also (103)) describes the identification and characterization of CAMP as a novel T1D-related factor. Expression of the *Camp* gene was induced in the jejunum of diet-protected HC-fed BBdp rats. CAMP co-localized with anti-inflammatory $CD163^{+}$ M2 macrophages, which we previously reported were increased in diet-protected rats (103). Unexpectedly, CAMP was detected in PLN and islets, including a subset of β-cells. In the pancreas of asymptomatic BBdp rats, there was a positive correlation between islet-associated CAMP expression and total islet number, indicating an association with islet expansion. In a pilot study, CAMP/LL-37 injections in young BBdp rats increased the abundance of probiotic and diabetes-protective bacteria and also promoted islet neogenesis. Thus, CAMP is an innate immune factor linking mucosal defenses, pancreatic immunity and islet biology.

RESEARCH OVERVIEW

Rationale

Diabetes-modifying diets altered the intestinal immune phenotype of BBdp rats. Weaning onto a cereal-based diet stimulated T cell infiltration and promoted T_H1-biased gene expression. Conversely, HC feeding promoted an expansion of anti-inflammatory $CD163^+$ M2 macrophages and *Cd163* transcription. Screening of immune genes could identify additional candidate factors involved in the dietary modification of inflammation in the jejunum of BBdp rats.

Hypotheses

(i) Dietary influence on small intestinal immunity in BBdp rats involves modification of innate and/or adaptive immune factors that can be identified by screening for altered expression of immune-associated genes; (ii) The cathelicidin antimicrobial peptide (CAMP) is a novel diabetes-inhibitory factor with beneficial effects on intestinal microbes, gut and pancreatic immunity, and islet homeostasis.

Research Questions

1. *Can novel diet-modifiable immune factors implicated in T1D pathogenesis be identified by gene expression screening in the jejunum of BBdp rats?*

2. *What cell types produce CAMP in BBdp rats?*

3. *Is pancreatic CAMP altered by inflammation?*

4. *Do CAMP/LL-37 injections in BBdp rats alter gut microbes, intestinal immune phenotype, and islet biology?*

RESULTS

Identification of novel diet-modifiable immune factors in the jejunum of 130 d BBdp rats

PCR Arrays for rat "Innate and Adaptive Immune Responses" (PARN-052A; Qiagen/SABiosciences, Mississauga, Canada) were used for screening of differential gene expression in jejunum of 130 d SPF-housed BBdp rats fed either a cereal or HC diet. The arrays contained primers for 84 immune genes, including T cell activation factors, cytokines, chemokines, pattern recognition receptors, and host defense factors. Four arrays were used for each diet group. Results are presented as a volcano plot (**Fig. 5.1A**). Compared with gene expression in jejunum of cereal-fed rats (control group), a value ≥ -2 fold identified HC-downregulated genes and a value of ≥ +2 fold was used as a threshold for identification of HC-upregulated genes.

HC-downregulated candidates:

Lck: *Lck* expression was 2.4-fold downregulated in HC-fed rats compared with cereal-fed rats (p=0.003; **Fig. 5.1B**). *Lck* encodes the lymphocyte-specific tyrosine kinase/p56, a src-family kinase involved in the initiation of T cell activation pathways upon TCR/co-receptor engagement (218). Downregulation of *Lck* in HC-fed rats suggests that a major intestinal effect of the protective diet was to inhibit T cell activation potential. This finding expands on our previous findings describing inhibition of jejunal T cell infiltration and decreased T_H1-biased transcription in HC-fed rats compared with cereal-fed counterparts (*see Chapter 3* and (103)). Thus, HC-downregulated *Lck* is supportive of the interpretation that HC feeding is associated with inhibition of T cell activation capacity in the jejunum of BBdp rats (103).

HC-upregulated candidates:

Il1f6: *Il1f6* expression was 2.8-fold upregulated in HC-fed rats compared with cereal-fed rats (p=0.004; **Fig. 5.1C**). *Il1f6* (also known as *Il36a*) encodes the cytokine interleukin 1 family member 6, which was previously characterized in skin and epithelial surfaces and found to be implicated in NF-κB signalling (219; 220). In transgenic mice over-expressing *Il1f6* in basal keratinocytes, development of neonatal inflammation was observed in the dermal and epidermal layers (220). In addition, pronounced expression of *Il1f6* was observed in skin lesions from patients with psoriasis whereas none was detected in normal skin tissue (220). IL-1F6 was reported to stimulate expression of genes encoding various antimicrobial peptides (221). In the present study, induction of *Il1f6* was observed in parallel with upregulation of CAMP.

Camp: *Camp* expression was 2.4-fold upregulated in HC-fed rats compared with cereal-fed rats (p=0.03; **Fig. 5.1D**). As described in this *Chapter's* introduction, CAMP is a major multifunctional host defense factor with roles including antimicrobial function, immunomodulation and tissue repair (222-224). CAMP was selected as a candidate factor for further exploration and characterization based on an interesting diversity of physiological effects (223) and the lack of information in the literature about this factor pertaining to T1D.

Validation of the candidate gene Camp as a factor upregulated by a protective HC diet

The jejunal upregulation of the *Camp* gene by HC feeding identified by immune transcriptional profiling was validated in another experiment.

FIGURE. 5.1

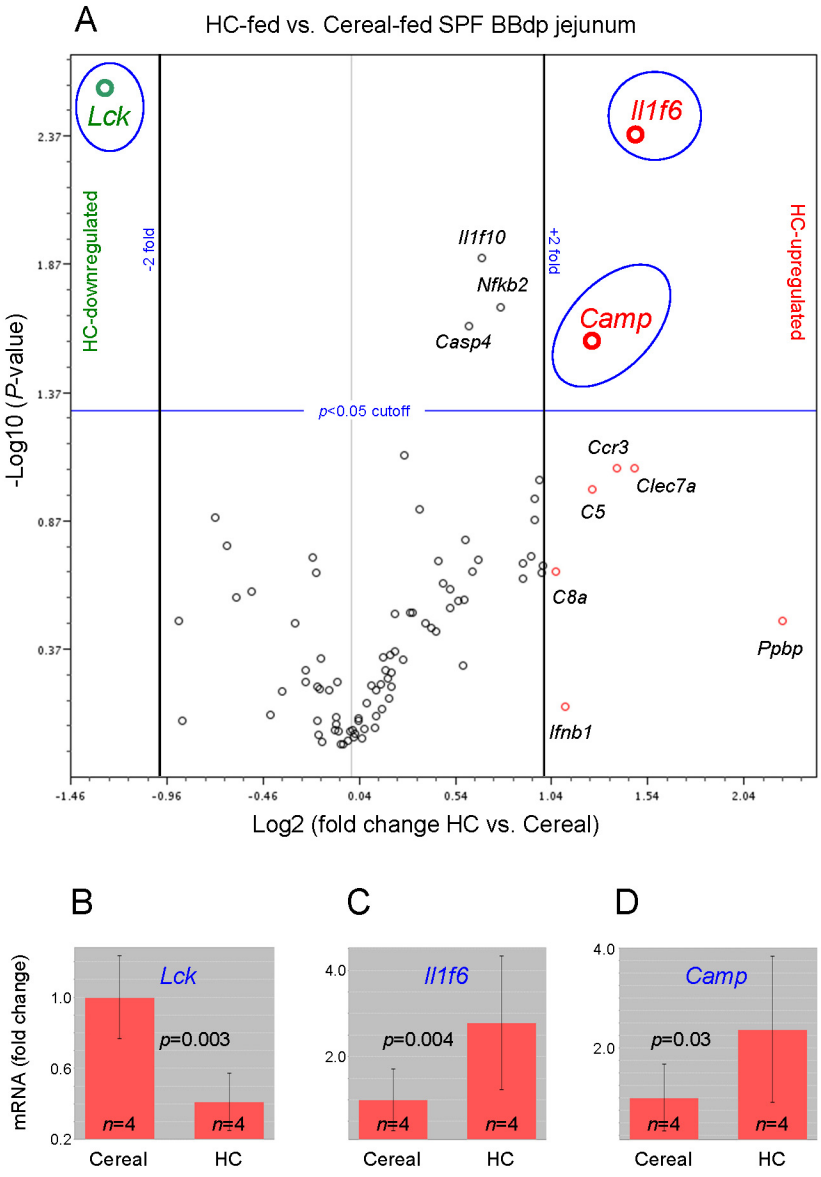

A
HC-fed vs. Cereal-fed SPF BBdp jejunum
Lck
Il1f6
Camp
Il1f10
Nfkb2
Casp4
Ccr3
Clec7a
C5
C8a
Ppbp
Ifnb1
HC-downregulated
-2 fold
+2 fold
HC-upregulated
p<0.05 cutoff
-Log10 (P-value)
Log2 (fold change HC vs. Cereal)
B
C
D
mRNA (fold change)
p=0.003
p=0.004
p=0.03
n=4
Cereal
HC

Figure 5.1. Transcriptional profiling of diet-modifiable immune genes in jejunum of BBdp rats

BBdp rats (130 d) were maintained under SPF conditions and fed either an HC diet or cereal diet and screened for innate and adaptive immune factors using PCR Arrays (n=4 arrays/dietary group). (**A**) Volcano plot displaying differential gene expression values and p-values; circled candidates were either HC-downregulated (green; *Lck*) or HC-upregulated (red; *Il1f6*, *Camp*) at least 2 fold with p-values < 0.05. Ct values for immune genes were normalized with the average Ct values of five housekeeping genes included in each array. Results are shown as fold change ($2^{-\Delta\Delta Ct}$) comparing expression in HC-fed rats relative to cereal-fed counterparts. Additional candidates (non-circled) are also indicated; these were either modified less than 2 fold or not statistically different. Summary results indicating dietary alteration in transcription of identified candidate factors (**B**) *Lck*, (**C**) *Il1f6*, and (**D**) *Camp*; p-values obtained using Student's t-test. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

Evaluation of *Camp* gene expression was repeated using an expanded number of samples and a different approach; RT-qPCR analysis was performed using a rat *Camp*-specific TaqMan Gene Expression Assay (Applied Biosystems, Life Technologies, Burlington, Canada). In keeping with PCR Array data, the analysis confirmed that HC-fed BBdp rats displayed significantly increased *Camp* transcription in the jejunum compared with cereal-fed counterparts (**Fig. 5.2A**). Thus, *Camp* was validated as a novel gene associated with dietary protection from T1D in the jejunum of BBdp rats. Involvement of intestinal CAMP – including its alteration by diet – has not been reported in T1D.

Characterization of CAMP protein in the small intestine of BBdp rats

Immunohistochemistry analysis was performed to characterize CAMP protein distribution in the jejunum of 130 d SPF-housed BBdp rats (**Fig. 5.2B**). The most pronounced expression of CAMP protein was observed in a subset of cells associated with the intestinal epithelium, which resembled epithelial-associated $CD163^+$ M2 macrophages (*see Chapter 3*). A more abundant population of $CAMP^+$ cells was observed in the lamina propria but staining intensity was relatively weaker compared with the aforementioned cells. As inferred by the protein name, CAMP is most strongly associated with antimicrobial defenses, despite displaying a spectrum of multifunctional activities. To determine whether CAMP production in the BBdp jejunum was dependent on microbial exposure or infection, intestinal tissue from two distinct sterile conditions was analyzed. CAMP was observed in jejunum from 130 d germ-free BBdp rats and in embryonic BBdp rats. These findings established that CAMP production in the BBdp jejunum did not require microbes and suggested potential involvement in intestinal homeostasis beyond antimicrobial function.

FIGURE. 5.2

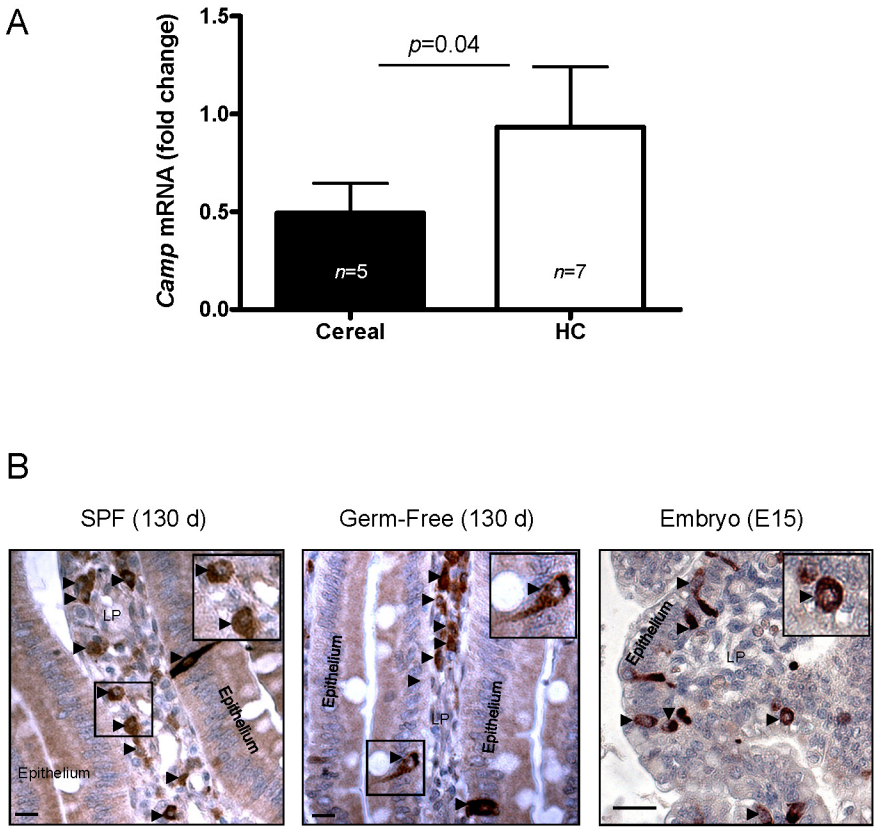

A
Camp mRNA (fold change)
1.5
1.0
0.5
0.0
p=0.04
n=5
n=7
Cereal
HC
B
SPF (130 d)
Germ-Free (130 d)
Embryo (E15)
LP
Epithelium
Epithelium
Epithelium
LP
Epithelium
Epithelium
LP

Figure 5.2. Characterization of CAMP protein distribution in jejunum of BBdp rats
(**A**) Small intestinal mRNA expression of *Camp* in jejunum of 130 d SPF-housed BBdp rats fed either cereal (filled bar) or HC (open bar). RT-qPCR performed using *Camp*-specific primers and probes from TaqMan Gene Expression Assay (Applied Biosystems, Life Technologies, Burlington, Canada)). *n*=5-7/group. Data represent mean ± SD; *p*-value obtained using Student's *t*-test. (**B**) Representative images displaying $CAMP^{+}$ cells (arrowheads) in lamina propria and epithelium of 130 d asymptomatic BBdp jejunum (left), bar=20 μm; 130 d germ-free BBdp jejunum (center), bar = 20 μm; and embryonic (E15) BBdp intestine (right), bar=25 μm. *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

Besides direct antimicrobial protection, other CAMP functions, such as immunomodulation and tissue repair, could be essential in the gastrointestinal tract, as suggested by abundant protein expression even under sterile conditions.

CAMP enriched in anti-inflammatory CD163$^+$ M2 macrophages in the BBdp rat jejunum

The profile of CAMP expression in the jejunum, featuring both epithelial-associated cells and lamina propria-associated cells, resembled the distribution pattern of anti-inflammatory CD163$^+$ M2 macrophages (103). Intestinal CAMP co-localized with CD163 and CD14 in BBdp jejunum (**Fig. 5.3A**) but not with CD68 (**Fig. 5.3B**), sometimes considered a marker of M1 macrophages (225; 226). CD163 is often classified as a specific marker of M2c macrophages (110; 111), a subset of M2 macrophages involved in various homeostatic processes including wound healing, tissue repair, and immunomodulation (113). Thus, CAMP was enriched in M2 macrophages and restricted to the M2c subtype based on additional co-localization with CD14, also enriched in the M2c phenotype (110; 113).

Discovery of CAMP in pancreatic lymph nodes

CAMP production is predominantly associated with host defenses at mucosal sites and skin (214). Unexpectedly, CAMP$^+$ cells were observed in a large subset of immune cells in pancreatic lymph nodes (PLN) of BBdp rats, even under germ-free conditions (**Fig. 5.4A**). CAMP presence in PLN has not been reported. CAMP$^+$ cells in PLN co-localized with a major subset of CD163$^+$ cells (**Fig. 5.4B**), consistent with jejunum findings (**Fig. 5.3A**). In addition, co-localization of PLN-associated CAMP was observed with CD14 (**Fig. 5.4C**), also consistent with the jejunum.

FIGURE. 5.3

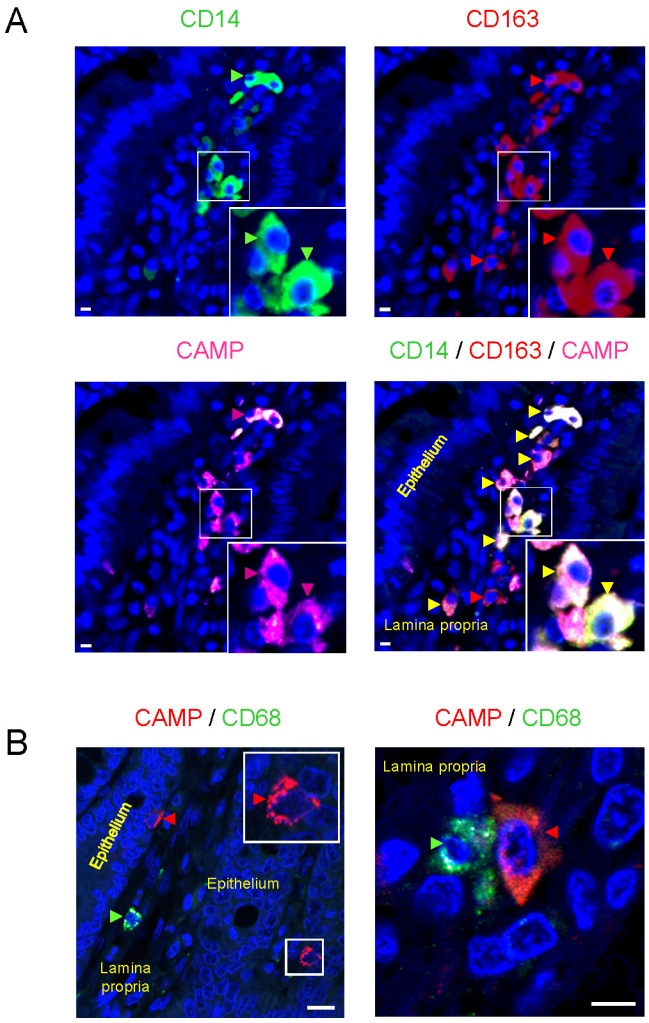

A
CD14
CD163
CAMP
CD14 / CD163 / CAMP
Epithelium
Lamina propria
B
CAMP / CD68
CAMP / CD68
Epithelium
Epithelium
Lamina propria
Lamina propria

Figure 5.3. CAMP production by M2 macrophages in jejunum of BBdp rats
(**A**) Representative immunofluorescence image obtained by confocal microscopy displaying $CAMP^+$ cells (magenta) in the lamina propria of 130 d asymptomatic germ-free BBdp jejunum co-localizing with CD14 (green arrowheads) and CD163 (red arrowheads); nuclear labelling by Hoechst; bars=5 μm. Yellow arrowheads in lower right panel indicate triple-positive population of $CAMP^+CD14^+CD163^+$ cells. (**B**) Representative confocal images displaying $CAMP^+$ cells (red arrowheads) adjacent to $CD68^+$ cells (green arrowheads) in lamina propria of 130 d asymptomatic germ-free BBdp jejunum; bars=20 μm (left panel) and 5 μm (right panel). *Note*: Figure modified and reprinted from material published in C. Patrick *et al*., *Diabetes* 2013 (103), with permission from the American Diabetes Association.

FIGURE. 5.4

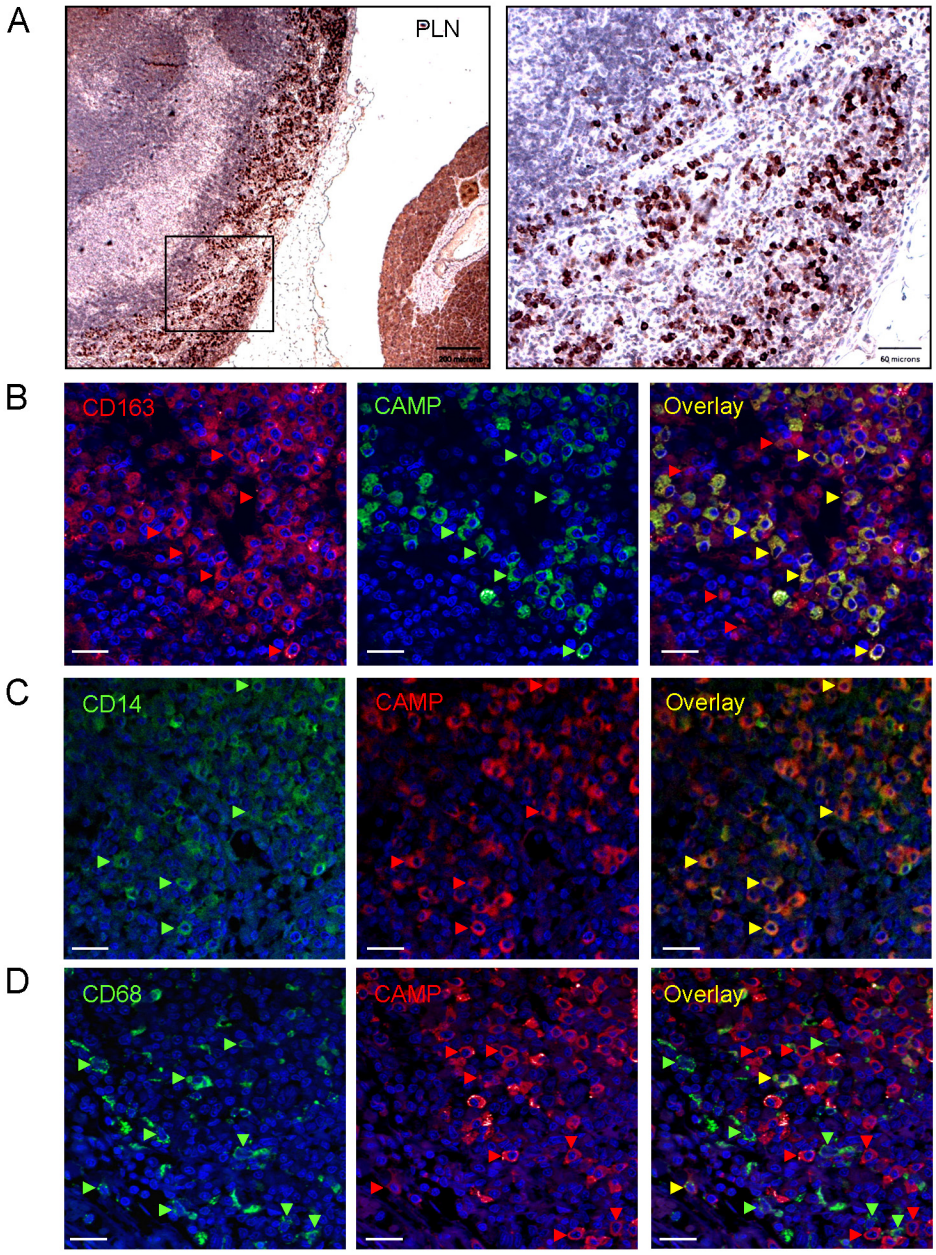

A
PLN
B
CD163
CAMP
Overlay
C
CD14
CAMP
Overlay
D
CD68
CAMP
Overlay

Figure 5.4. Discovery of CAMP in PLN of BBdp rats

(**A**) Representative image displaying $CAMP^+$ cells in PLN from a 130 d asymptomatic germ-free BBdp rat; bar=200 μm (left panel); bar=50 μm (right panel). (**B**) Representative immunofluorescence image obtained by confocal microscopy displaying a large subset of $CAMP^+$ cells (green arrowheads) co-localizing with CD163 (red arrowheads) in PLN; bars=20 μm. Yellow arrowheads indicate co-localized $CAMP^+CD163^+$ cells. (**C**) Representative confocal image displaying $CAMP^+$ cells (red arrowheads) co-localizing with CD14 (green arrowheads) in PLN; bars=20 μm. Yellow arrowheads indicate co-localized $CAMP^+CD14^+$ cells. (**D**) Representative confocal image displaying $CAMP^+$ cells (red) adjacent to CD68 (green) in PLN; bars=20 μm. Yellow arrowheads indicate minor subset of co-localized $CAMP^+CD68^+$ cells.

Therefore, PLN-associated CAMP was present in a large subset of $CD163^+$ M2 macrophages and potentially in monocytes. CAMP rarely co-localized with CD68 in PLN (**Fig. 5.4D**), again in keeping with jejunum results. Interestingly, CAMP did not co-localize with CD163 in the spleen (not shown), suggesting that antimicrobial production by $CD163^+$ M2 macrophages was restricted to cells in the gastrointestinal tract. Thus, M2 macrophages co-expressing CAMP were present in both jejunum and PLN, suggesting that $CD163^+CD14^+CAMP^+$ cells represent a previously unrecognized population that participates in small intestinal and pancreatic immunity.

Discovery of CAMP in pancreatic islets

Besides our report (103), dietary modification of CAMP by cereal or HC has not been reported in the literature, nor has enrichment in M2 macrophages or expression in PLN. Most surprisingly, I serendipitously discovered $CAMP^+$ cells in pancreatic islets. Additional studies revealed CAMP was present in islets from both adult germ-free BBdp rats (**Fig. 5.5A**) and BBdp embryos (**Fig. 5.5B**). In the embryonic pancreas, there was a close association between $CAMP^+$ cells and $insulin^+$ cells. Embryonic CAMP expression suggests a novel role in islet formation; this interpretation was supported by subsequent detection of CAMP in a subset of neogenic tubular complexes (not shown), which are unique structures considered foci of β-cell regeneration (227). Islet-associated $CAMP^+$ cells were also present in SPF-housed BBdp rats (**Fig. 5.6A**). In addition to BB rats, islet-associated CAMP was also observed in a variety of different rat strains, including Lewis rats, Komeda rats, Wistar Furth rats, and Sprague-Dawley rats (not shown). With the notable exception of embryonic BBdp islets displaying abundant CAMP, detection of the peptide in islet cells appeared to be less frequent in younger BBdp rats (~14-50 d) compared with older animals (~60-150 d) (not

quantified). Among asymptomatic 130 d BBdp rats, there was a significant positive correlation between the number of islet-associated CAMP$^+$ cells and total islet number per pancreas (**Fig. 5.6B**). When islet-associated CAMP$^+$ cells were quantified in ~100-130 d animals, asymptomatic BBdp rats had a significantly greater number of islet-associated CAMP$^+$ cells compared with BBc rats (**Fig. 5.6C**). Also, the number of CAMP$^+$ cells was highest in islet remnants from overtly diabetic BBdp rats, which was significantly increased compared with islets from asymptomatic counterparts (**Fig. 5.6C**). In inflamed islets of asymptomatic 130 d BBdp rats, CAMP$^+$ cells appeared in the vicinity of islet-infiltrating pro-inflammatory CD68$^+$ macrophages, but these factors did not co-localize (**Fig. 5.7A**), consistent with jejunum and PLN observations. In islet remnants from overtly diabetic BBdp rats, CAMP$^+$ cells were again found in close proximity to infiltrating CD68$^+$ macrophages as well as residual insulin$^+$ cells (**Fig. 5.7B**). Thus, CAMP continues to be expressed in the target tissue even during inflammatory and hyperglycaemic states.

Human pancreas samples from autopsies of patients with T1D were also examined for the presence of CAMP. The samples were from male and female patients aged 6-27, mostly recent-onset T1D obtained during the 1920s (samples supplied by Dr. A.K. Foulis, Royal Infirmary, Glasgow, UK). Of note, human CAMP$^+$ cells were abundant in islet remnants from patients with T1D (**Fig. 5.7C**) (Dr. G.-S. Wang, Scott lab), consistent with BBdp rats. Thus, CAMP production is a feature of embryonic islet development in BBdp rats, and expression of this host defence factor can become enriched postnatally under local inflammatory conditions.

In BBdp rats, CAMP was associated with enhanced islet number in the asymptomatic state and appeared adjacent to $CD68^+$ cells during insulitis, possibly as part of a counter-inflammatory immune response to β-cell autoimmunity. Interestingly, in a 100 d asymptomatic BBdp rat without insulitis, many endocrine cells within the islets appeared to express CAMP (**Fig. 5.8A**). Conversely, endocrine-associated CAMP staining was relatively weak in an age-matched asymptomatic BBdp counterpart displaying insulitis (**Fig. 5.8B**). However, in insulitic islets, $CAMP^+$ cells were abundant among the islet-associated immune infiltrate, resembling the original findings obtained using asymptomatic germ-free BBdp rats containing inflamed islets (**Fig. 5.5A**). Thus, the source of islet-associated CAMP can originate from either endocrine or immune cells, depending on the local islet inflammatory status. Both endocrine-associated and immune-associated $CAMP^+$ populations were clearly observed in parallel in inflamed islets of asymptomatic NOD mice (**Fig. 5.8C**), illustrating the distinct dual localization of this peptide in islets from rodent models of T1D.

FIGURE. 5.5

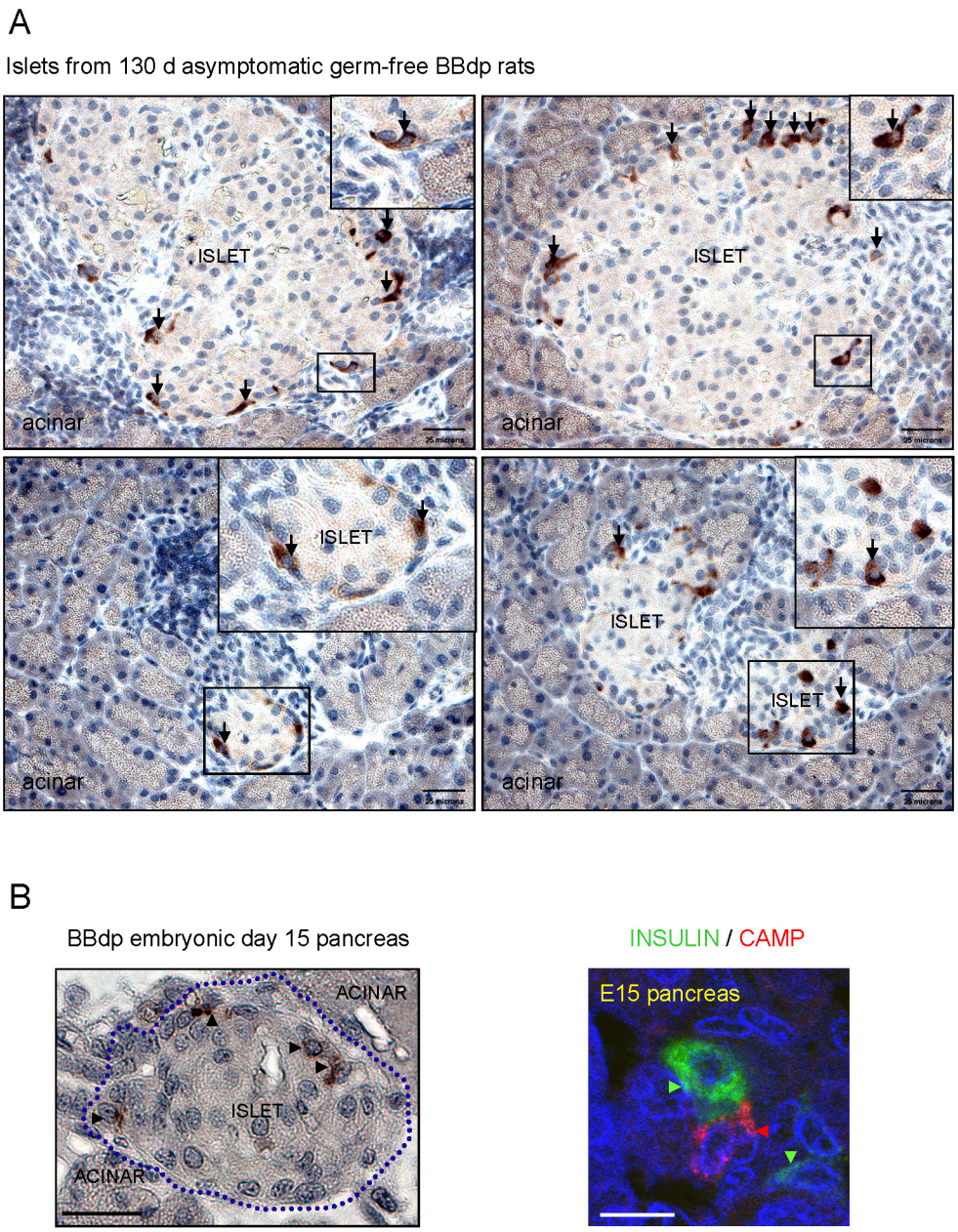

A
Islets from 130 d asymptomatic germ-free BBdp rats
ISLET
acinar
ISLET
acinar
ISLET
acinar
ISLET
ISLET
acinar
B
BBdp embryonic day 15 pancreas
ACINAR
ISLET
ACINAR
INSULIN / CAMP
E15 pancreas

Figure 5.5. Discovery of CAMP in islets of BBdp rats

$CAMP^+$ cells were observed in pancreatic islets from BBdp rats, even under sterile conditions. (**A**) Representative images displaying multiple $CAMP^+$ cells (arrows) within various islets of 130 d asymptomatic germ-free BBdp rats; bars=25 μm. $CAMP^+$ cells were more prevalent at the islet periphery. (**B**) Representative image of developing islet in BBdp rat embryo at embryonic day 15 (E15, left panel); bar=25 μm. Immunofluorescence image obtained by confocal microscopy displaying $CAMP^+$ cell (red arrowhead) adjacent to $insulin^+$ cells (green arrowheads) in embryonic BBdp pancreas; bar=10 μm.

FIGURE. 5.6

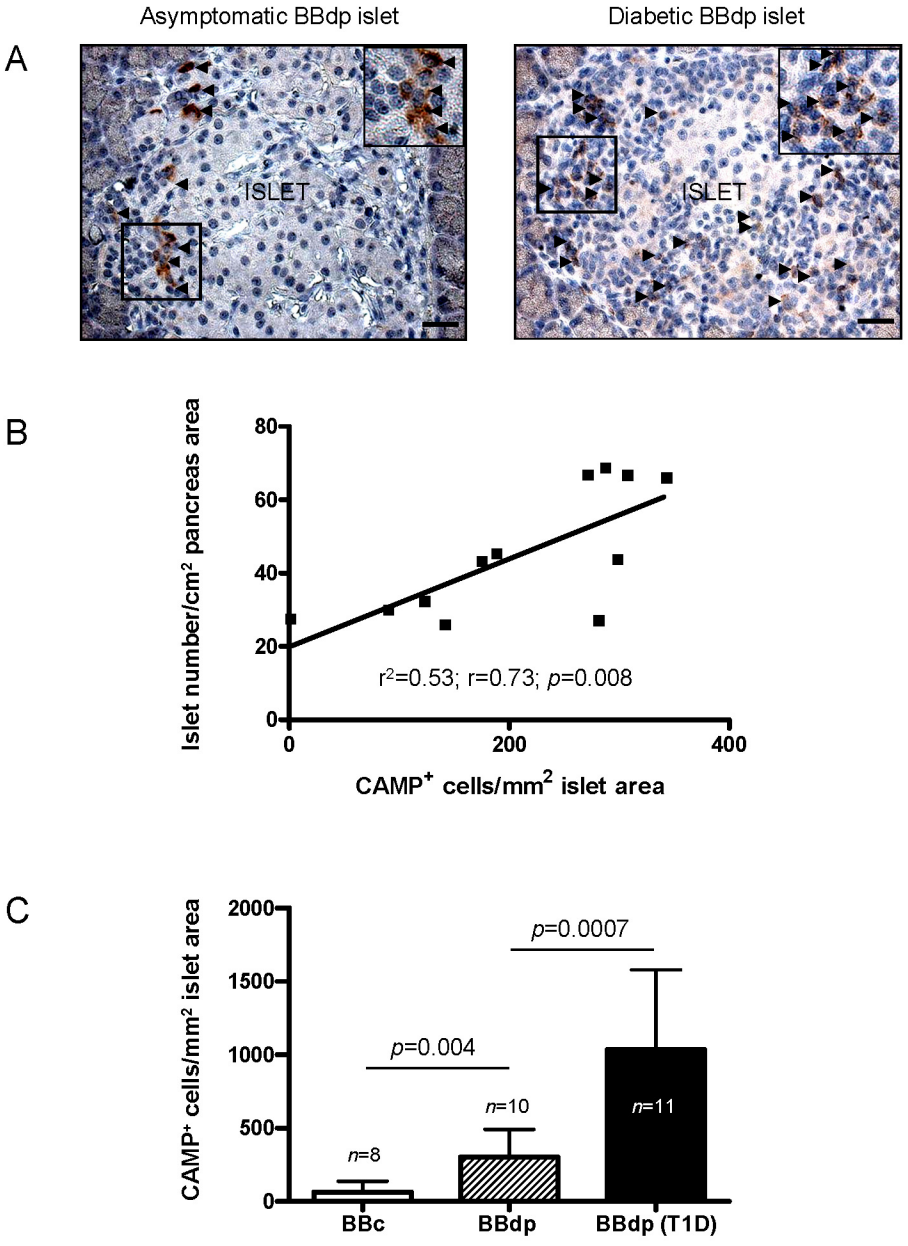

A
Asymptomatic BBdp islet
Diabetic BBdp islet
ISLET
ISLET
B
Islet number/cm² pancreas area
80
60
40
20
0
0
200
400
r²=0.53; r=0.73; p=0.008
CAMP⁺ cells/mm² islet area
C
CAMP⁺ cells/mm² islet area
2000
1500
1000
500
0
p=0.0007
p=0.004
n=8
n=10
n=11
BBc
BBdp
BBdp (T1D)

Figure 5.6. CAMP in islets associated with islet number and altered by inflammatory status

Islet-associated $CAMP^+$ cells were also observed in SPF-housed BBdp rats. **(A)** Representative images displaying $CAMP^+$ cells (arrowheads) within islets of ~100 d asymptomatic BBdp rat (left panel) and overt diabetic BBdp rat; bars=25 μm. (**B**) Significant positive correlation between density of islet-associated $CAMP^+$ cells and total islet number per pancreas among ~100 d asymptomatic BBdp rats fed a cereal diet or HC diet. *n*=12. Correlation coefficient obtained using Pearson's correlation; *p*-value obtained by linear regression analysis. (**C**) Density of islet-associated $CAMP^+$ cells in pancreas of ~100 d control (BBc) rats (open bar), asymptomatic BBdp rats (hatched bar), and overtly diabetic BBdp rats (filled bar). *n*=8-11/group; data represent mean ± SD. *P*-values obtained using Student's *t*-test.

FIGURE. 5.7

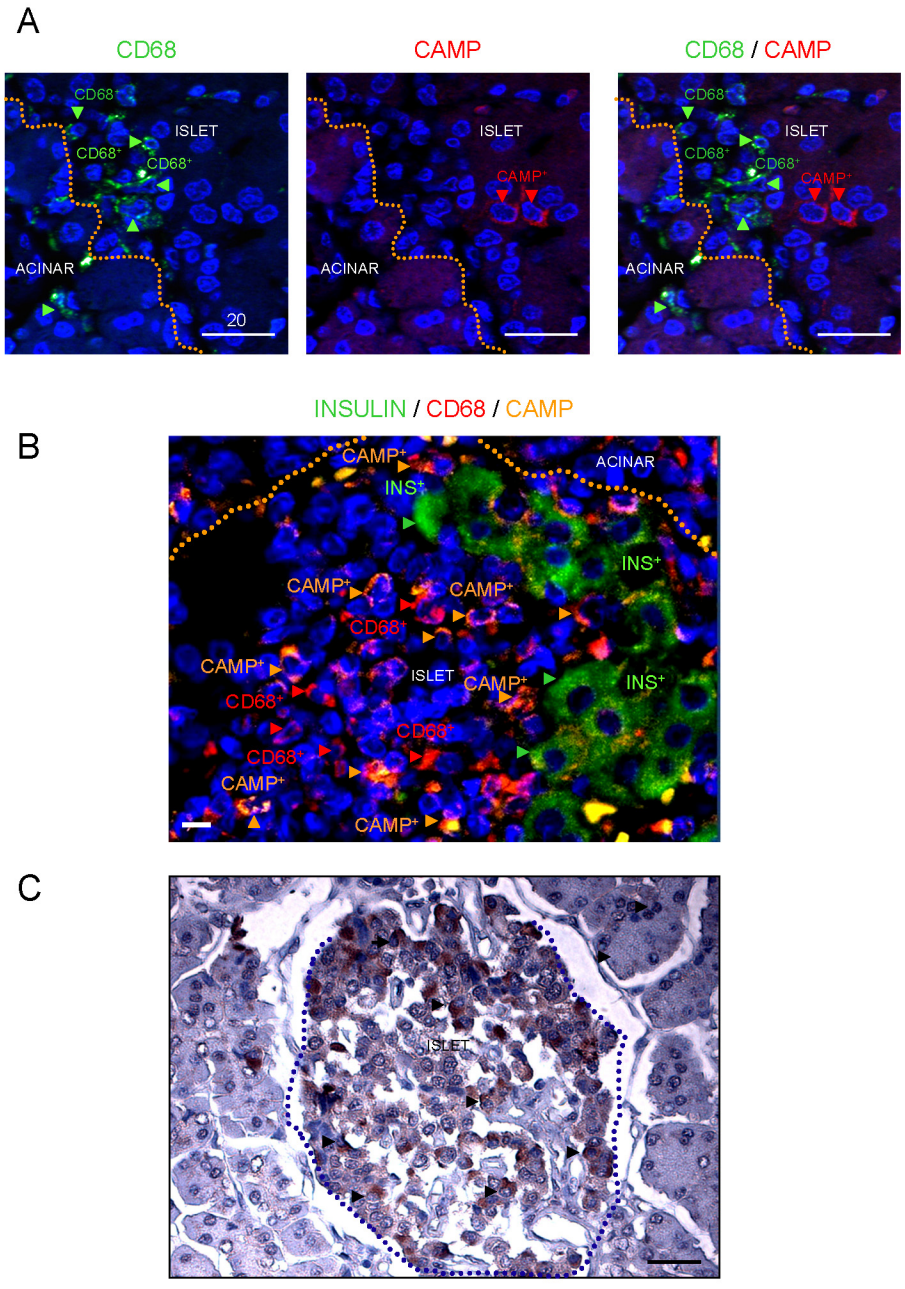


Figure 5.7. Characterization of CAMP$^+$ cells in inflamed islets of BBdp rats and humans

(**A**) Representative immunofluorescence image obtained by confocal microscopy displaying CAMP$^+$ cells (red arrowheads) in the vicinity of CD68$^+$ cells (green arrowheads) in insulitic islet (dotted outline) from asymptomatic 130 d germ-free BBdp rat; bars=20 μm. (**B**) CAMP$^+$ cells (orange arrowheads) are shown in the vicinity of CD68$^+$ cells (red arrowheads) and insulin$^+$ cells (green arrowheads) in islet remnant (dotted outline) of overtly diabetic BBdp rat; bar=10 μm. (**C**) Multiple CAMP$^+$ cells (arrowheads) within islet remnant (dotted outline) of a 12 year-old female patient with recent-onset T1D (case# E336, Dr. A.K. Foulis, see below); bar=25 μm. *Note*: Staining and image courtesy Dr. G.-S. Wang, Ottawa Hospital Research Institute, Ottawa, Canada; human pancreas tissue courtesy of Dr. A.K. Foulis, Royal Infirmary, Glasgow, UK.

FIGURE. 5.8

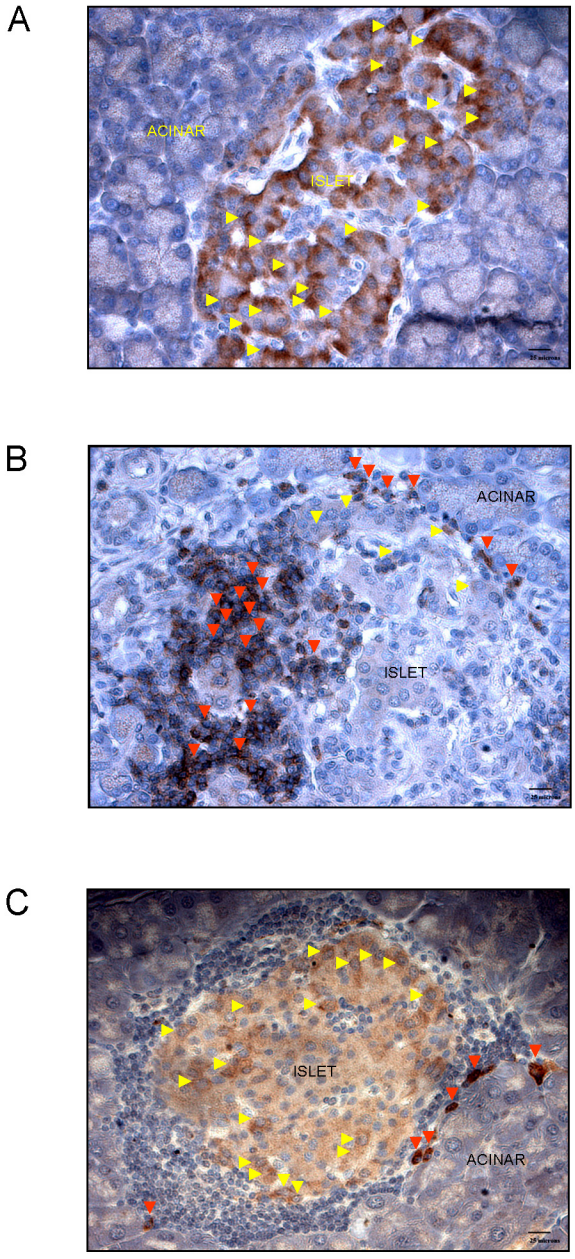


Figure 5.8. Distinct sources of islet-associated CAMP dependent on inflammation
(**A**) Representative image of non-inflamed islet from 100 d asymptomatic BBdp rat displaying widely distributed CAMP staining (yellow arrowheads) throughout islet endocrine cells; bar=25 μm. (**B**) Representative image of inflamed islet from 100 d asymptomatic BBdp rat displaying subset of $CAMP^+$ cells distributed among islet-associated immune infiltrate (orange arrowheads). Relatively weak CAMP staining was detected within a small subset of endocrine cells within the inflamed islet (yellow arrowheads); bar=25 μm. (**C**) Representative image of inflamed islet from NOD mouse displaying parallel expression of both islet-associated sources of CAMP; endocrine-based CAMP staining in islets (yellow arrowheads) and immune-associated $CAMP^+$ cells (orange arrowheads) at the islet periphery; bar=25 μm.

CAMP detection in β-cells in asymptomatic rats

CAMP appeared to be distributed in some endocrine cells, including β-cells, in non-inflamed states. To confirm β-cell-specific expression, CAMP co-localization experiments were performed with insulin. CAMP co-localized with a subset of insulin^{+} cells in adult SPF-housed BBdp rat islets (**Fig. 5.9A**), germ-free BBdp rat islets (**Fig. 5.9B**) and embryonic BBdp rat islets (**Fig. 5.9C**). CAMP has been shown to drive mammalian signalling processes in epithelial cells from skin (228), lungs (229), and cornea (230), playing an important role in growth and repair. In a cancer study, CAMP was reported to be a potent agonist of the insulin-like growth factor 1 receptor (IGF-1R) (231). Interestingly, IGF-1 itself is also known to induce *Camp* expression (232). In corneal cells, CAMP exposure was reported to stimulate phosphorylation of epidermal growth factor receptor (EGFR) and downstream effectors Akt and Erk (233). CAMP has not been described previously as a product of endocrine cells and its discovery in islets represents a novel aspect of islet biology and T1D.

Validation of CAMP expression in the pancreas

To verify whether *Camp* was transcribed in the pancreas, mRNA detection was performed by RT-qPCR using *Camp*-specific primers and probes (TaqMan Gene Expression Assay; Applied Biosystems, Life Technologies, Burlington, Canada). Amplification of *Camp* cDNA was observed using RNA extracted from whole BBdp jejunum (positive control; **Fig. 5.10A**), whole BBdp pancreas (**Fig. 5.10B**), and collagenase-isolated BBc islets (**Fig. 5.10C**). *Camp* expression was not observed in the INS-1E rat insulinoma β-cell line (data not shown). Thus, *Camp* mRNA detection in whole pancreas and isolated islets supports the view that CAMP is expressed in islets.

FIGURE. 5.9

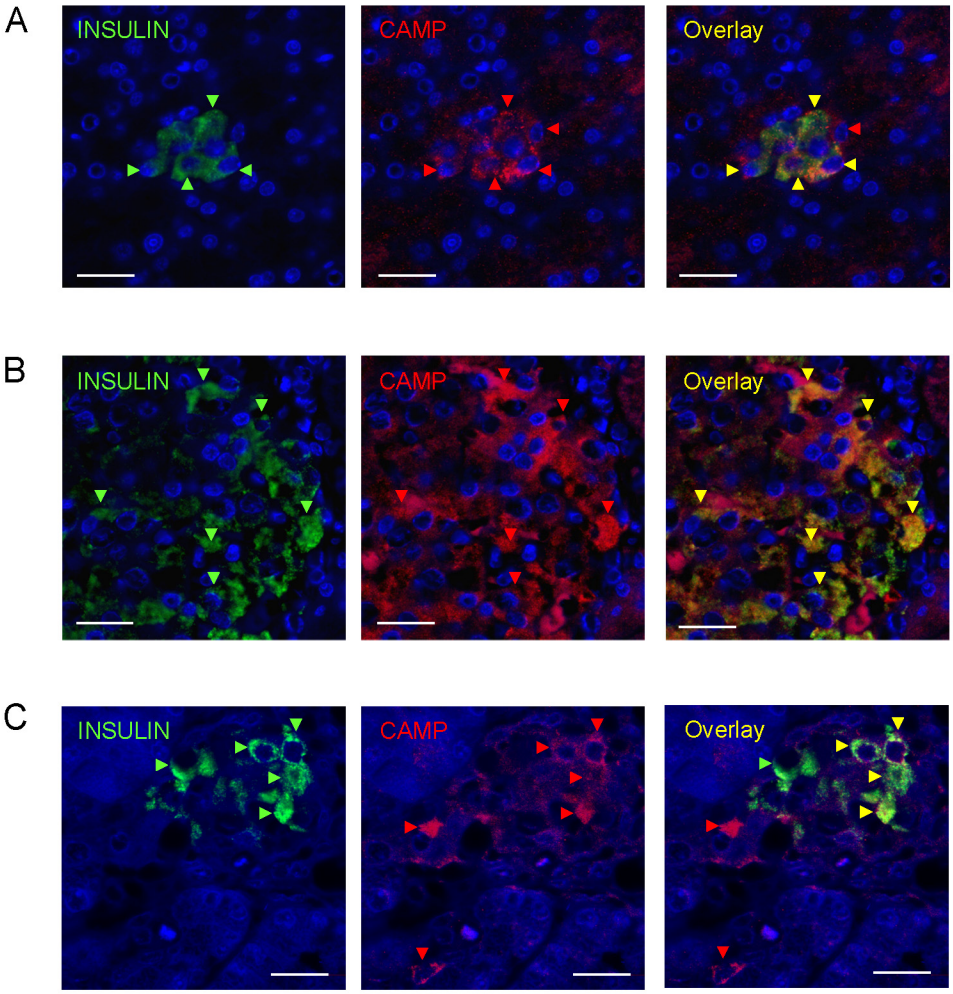

A
INSULIN
CAMP
Overlay
B
INSULIN
CAMP
Overlay
C
INSULIN
CAMP
Overlay

Figure 5.9. CAMP co-localization with insulin in non-inflamed islets of BBdp rats
In non-inflamed conditions, a subset of insulin^{+} cells co-localized with CAMP, in both the SPF condition and sterile states. Representative immunofluorescence images obtained by confocal microscopy displaying CAMP^{+} cells (red arrowheads) co-localizing with a subset of insulin^{+} cells (green arrowheads) in non-inflamed islets from (**A**) 150 d asymptomatic SPF-housed BBdp rat, (**B**) 130 d asymptomatic germ-free BBdp rat, and (**C**) BBdp embryo; bars=20 μm. Yellow arrowheads indicate subset of co-localized CAMP^{+}insulin^{+} cells.

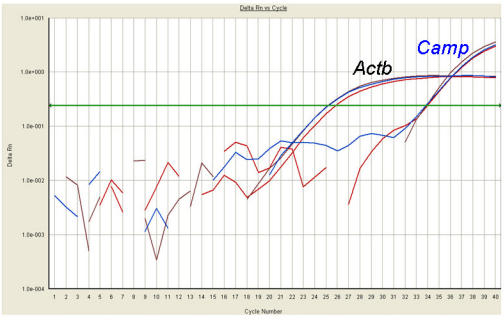
Effects of injected CAMP/LL-37 on gut immune homeostasis, gut bacteria, and islet biology

The immunomodulatory properties and tissue repair function of CAMP make this host defense peptide an attractive therapeutic factor for T1D. The natural distribution of CAMP in the gastrointestinal tract, PLN, and islets suggest that the endogenous protein is ideally situated to influence microbes, the immune system (gut, pancreas/PLN), and tissue homeostasis (gut, pancreas/islets). To explore the therapeutic potential of CAMP, exogenous human CAMP (also known as LL-37) was injected i.p. in young BBdp rats in a pilot study. The objectives were to determine whether CAMP/LL-37 administration could alter the small intestinal immune profile, influence the composition of gut microbial communities, and alter islet homeostasis. BBdp rats were weaned onto a cereal-based diet at 23 d. Beginning at 24 d, animals were injected daily for one week with either CAMP/LL-37 (1 mg/kg; *n*=10/group) or saline (*n*=10/group) (**Fig. 5.11A**). Following CAMP/LL-37 injections, there was a trend of increased $CD163^+$ M2 macrophages in the jejunum, which achieved statistical significance with removal of two identified outliers (**Fig. 5.11B**). Targeting gut microbes in susceptible subjects could be an effective strategy for T1D modulation (234). Fecal pellets were collected following treatment for bacterial analysis. Collaborators Dr. A. Stintzi and W. Mettawea (University of Ottawa, Ottawa, Canada) extracted bacterial DNA and performed high-throughput sequencing and bacterial analysis to determine the composition of the microbiota. BBdp rats injected with CAMP/LL-37 displayed an increased abundance of *Actinobacteria* (phylum and class classification), including an increased abundance of the *Bifidobacteriaceae* family (**Fig. 5.11C**). Fecal samples from BB-DP and resistant (BB-DR) rats were previously compared and a greater abundance of *Bifidobacterium* was reported in resistant animals (87). Also, oral administration of VSL#3, a probiotic blend including three species of *Bifidobacterium*, inhibited T1D in NOD mice (211). Thus, CAMP/LL-37

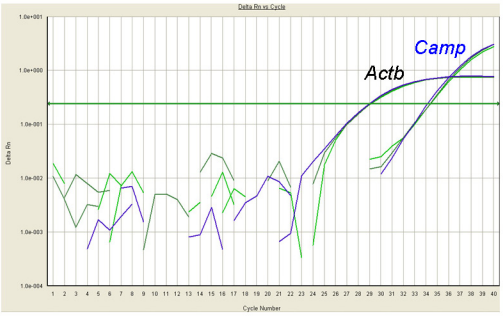
injections modified the microbiota of BBdp rats, with upregulation of probiotic bacteria. In addition, CAMP/LL-37-injected BBdp rats displayed an increased abundance of *Coriobacteriales* (order) and *Coriobacteriaceae* (family) compared with saline-injected controls; *Adlercreutzia* (genus), bacteria from the same phylogenetic lineage, were also more abundant in CAMP/LL-37-injected animals. Interestingly, *Coriobacteriales* were more abundant in 30 d fecal samples from diabetes-resistant BBdp rats compared with pre-diabetic BBdp rats (**Fig. 5.11D**) in a separate prospective study done in collaboration with Dr. A. Stintzi and T. Abujamel (University of Ottawa, Ottawa, Canada). In summary, CAMP/LL-37 injections in young BBdp rats upregulated probiotic bacteria (e.g. *Bifidobacteria*) as well as diabetes-protective *Coriobacteria*. Thus, potentially beneficial modification of microbiota in BBdp rats was achieved by administration of exogenous CAMP/LL-37. Interestingly, these findings could partly explain the mechanism by which HC feeding protected BBdp rats from T1D under SPF conditions, by upregulating antimicrobial production, altering intestinal bacterial composition and promoting islet neogenesis (not shown).

FIGURE. 5.10

A Jejunum (BBdp)



B Pancreas, whole (BBdp)



C Islet, collagenase-isolated (BBc)

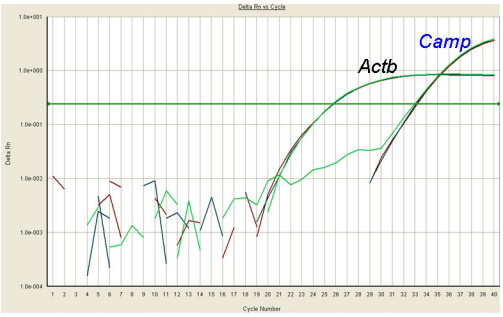


Figure 5.10. Validation of *Camp* expression in BB pancreas and islets
Camp gene expression by RT-qPCR using *Camp*-specific primers and probes from a TaqMan Gene Expression Assay (Applied Biosystems, Life Technologies, Burlington, Canada). Representative amplification plots of *Camp* cDNA in (**A**) BBdp jejunum, (**B**) BBdp whole pancreas, and (**C**) collagenase-isolated BBc islets. Multiplex qPCR amplification plots are shown, displaying detection of β-actin (*Actb*) and *Camp* mRNA; technical triplicates shown.

FIGURE. 5.11

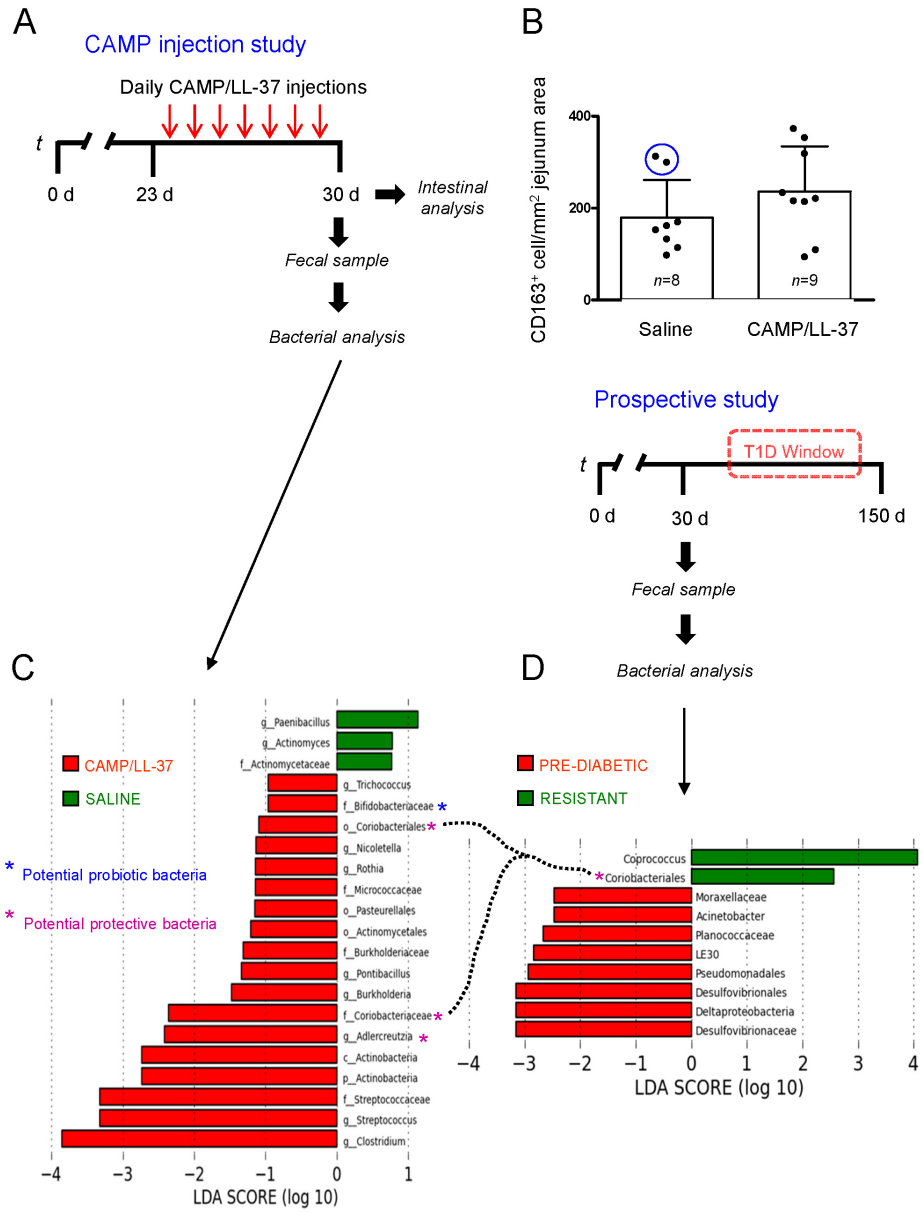

A
CAMP injection study
Daily CAMP/LL-37 injections
t
0 d
23 d
30 d
Intestinal analysis
Fecal sample
Bacterial analysis
B
CD163+ cell/mm2 jejunum area
400
200
0
n=8
n=9
Saline
CAMP/LL-37
Prospective study
T1D Window
t
0 d
30 d
150 d
Fecal sample
Bacterial analysis
C
CAMP/LL-37
SALINE
* Potential probiotic bacteria
* Potential protective bacteria
g_Paenibacillus
g_Actinomyces
f_Actinomycetaceae
g_Trichococcus
f_Bifidobacteriaceae *
o_Coriobacteriales *
g_Nicoletella
g_Rothia
f_Micrococcaceae
o_Pasteurellales
o_Actinomycetales
f_Burkholderiaceae
g_Pontibacillus
g_Burkholderia
f_Coriobacteriaceae *
g_Adlercreutzia *
c_Actinobacteria
p_Actinobacteria
f_Streptococcaceae
g_Streptococcus
g_Clostridium
−4 −3 −2 −1 0 1
LDA SCORE (log 10)
D
PRE-DIABETIC
RESISTANT
Coprococcus
*Coriobacteriales
Moraxellaceae
Acinetobacter
Planococcaceae
LE30
Pseudomonadales
Desulfovibrionales
Deltaproteobacteria
Desulfovibrionaceae
−4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)

Figure 5.11. CAMP/LL-37 injections modify the intestinal microbiota in young BBdp rats

Pilot study exploring the therapeutic potential of exogenous CAMP/LL-37 administration in young BBdp rats. (**A**) Schematic of experimental protocol; starting at 23 d (weaning), cereal-fed BBdp rats were administered daily intraperitoneal injections for 7 days with either CAMP/LL-37 (1 mg/kg; n=10) or saline (n=10) and killed at 30 d for investigation of short-term effects on immune cell distribution in the jejunum and composition of the fecal microbiota. (**B**) Number of $CD163^+$ cells in the jejunum of BBdp rats following injections of CAMP/LL-37 or saline. Data represent mean ± SD; n = 8-9 rats/group. p-value = 0.03 when circled outliers are removed; p-value obtained using Student's t-test. (**C**) Evaluation of differences in fecal microbiota from 30 d BBdp rats following treatment with CAMP/LL-37 or saline. Red = CAMP/LL-37 treatment; green = saline-injected. Histogram displays differentially abundant bacterial taxa identified by linear discriminant analysis (LDA); p<0.05, Kruskal-Wallis test. The linear discriminant effect size method (LEFSe) is a strategy for identification of "biological features explaining differences between groups" (235). Taxa identified by the LEFSe method explain the greatest differences between the microbial communities from the two groups (i.e. CAMP/LL-37-treated vs. saline-injected). (**D**) Evaluation of differences in fecal microbiota in diabetes-resistant 30 d BBdp rats compared with pre-diabetic BBdp rats. Fecal samples were retrospectively classified as either "diabetes-resistant" or "pre-diabetic" on the basis of subsequent T1D development as part of a prospective study that followed disease fate up until 150 d. Red = pre-diabetic; green = diabetes-resistant. Histogram displays differentially abundant taxa identified by LDA; p<0.05, Kruskal-Wallis test. *Note*: Bacterial analyses performed by Dr. A Stintzi, W. Mettawea, and T. Abujamel (University of Ottawa, Ottawa, Canada).

SUMMARY

A screening experiment identified the host defense factor *Camp* as a protective immune factor in the jejunum of HC-fed BBdp rats. Intestinal CAMP was enriched in anti-inflammatory $CD163^+$ M2 macrophages and $CAMP^+CD163^+$ cells were unexpectedly observed in PLN. CAMP was also detected in islets, including a subset of β-cells. CAMP expression correlated with islet number and was increased during inflammation. Thus, CAMP could represent a novel islet trophic factor supporting pancreatic homeostasis. CAMP/LL-37 injections increased the abundance of probiotic and diabetes-protective bacteria. CAMP represents a novel innate immune hub linking microbes, mucosal defenses, pancreatic immunity, and islet biology.

DISCUSSION

The objective of this study was to identify and characterize novel intestinal immune genes associated with the dietary modification of T1D in BBdp rats. Using a PCR Array immune-focused screening strategy, the *Camp* gene was identified as a novel innate immune factor upregulated in the jejunum of BBdp rats fed a protective HC diet. Thus, the protective upregulation of M2 macrophages observed in the jejunum of HC-fed rats (103) occurred in parallel with *Camp* induction. Interestingly, CAMP co-localized with CD163 in jejunum and PLN, even under sterile conditions, indicating that expression was independent of microbes and that other pleiotropic functions could be essential in the gastrointestinal tract. CAMP has been characterized as a product of various cell types, including epithelial cells, monocytes, macrophages, and neutrophils (236). However, the present work reveals that M2 macrophages are a predominant source of CAMP in the jejunum and PLN of BBdp rats. CAMP is multifunctional and major roles besides antimicrobial function include

immunomodulation, growth promotion, chemotaxis, wound healing, and angiogenesis (223). Many of these functions are also attributed to M2 macrophages (237). Thus, CAMP could represent an important effector peptide of M2 macrophages that directly contributes to the various homeostatic roles ascribed to these anti-inflammatory cells.

Studies using *Camp*-deficient mice have demonstrated the importance of this AMP in anti-bacterial and anti-viral immunity in various tissues including skin, urinary tract, eye, and gut (238-242). Altered CAMP status has also been described in the context of inflammatory bowel diseases (243; 244). For example, decreased CAMP was reported in patients with Crohn's disease (245). Also, Koon *et al*. reported that CAMP expression by monocytes contributed to inhibition of colitis in mice (246). Thus, sufficient production of CAMP in the gut by $CD163^{+}$ macrophages could similarly be important in maintaining intestinal homeostasis and inhibiting diabetes-associated inflammation. Antimicrobial deficiency can exacerbate intestinal inflammation caused by non-microbial insults. For example, *Camp*-deficient mice developed more pronounced colitis upon exposure to the pro-colitic chemical dextran sodium sulphate (DSS) (246). By analogy, if pro-inflammatory diabetes-promoting cereal antigens were present in the context of local CAMP deficiency, intestinal inflammation could be exacerbated, as bacterial interaction with host tissue increased. In *Chapter 3*, diet-protected HC-fed BBdp rats had decreased T cell infiltration in the epithelium and lamina propria which was associated with increased $CD163^{+}$ macrophages and *Cd163* transcription (103). In the present study, HC feeding promoted *Camp* expression, which could have limited direct bacterial interaction with the gut epithelium resulting in inhibition of gut infiltration by IEL and LPL. In support of this interpretation, a significant HC-associated decrease in jejunal expression of the T cell activation factor *Lck* was observed

in parallel with *Camp* induction during the screening experiment. Thus, CAMP could be influential at the gut epithelial surface, as it could inhibit bacterial interaction with the host while limiting inflammation via anti-inflammatory pathways.

Although BBdp rats lacking microbes can ultimately develop T1D (103), altered bacterial composition is associated with T1D. In a study by Roesch *et al*., fecal bacteria from diabetes-resistant BB-DR rats were compared with BB-DP samples (87). Overall, probiotic species from the *Lactobacillus* and *Bifidobacteria* genera were more abundant in the BB-DR strain, indicating an association with T1D protection (87). Direct administration of a *Lactobacillus* species inhibited T1D in BB-DP rats (88) and a similar effect was reported in NOD mice using the probiotic mixture VSL#3 (211; 212). In both models, the anti-diabetic effects of probiotics were associated with decreased pro-inflammatory gut immune activation (88; 211). Young BBdp rats injected with CAMP/LL-37 displayed increased abundance of probiotic bacteria and diabetes-protective bacteria, including *Bifidobacteria* and *Coriobacteria*, indicating that administration of this factor enriched beneficial microbes. In humans, the combined abundance of two *Bifidobacterium* species, *B. adolescentis* and *B. pseudocatenulatum*, was decreased in autoantibody-positive individuals (90); high-risk individuals with four autoantibodies displayed decreased numbers of butyrate-producing bacteria (90). Butyrate is a potent inducer of *Camp* expression (247). Thus, there is a potential interplay among AMPs, microbes, and intestinal inflammation, which could represent a target for intervention in T1D. CAMP administration intra-colonically prevented colitis in mice, an effect associated with bacterial reduction and decreased immune cell infiltration (248), demonstrating the concomitant microbe-modifying and immune-modifying

effects of this peptide. CAMP/LL-37 injections enhanced the abundance of probiotic bacteria, supporting a role in intestinal homeostasis.

CAMP staining in PLN was enriched in $CD163^+$ M2 macrophages, which are ideally situated for modulation of pancreatic autoimmunity. CAMP detection in lymph nodes is rarely reported in the literature, with only one study in pigs describing the porcine cathelicidin PR-39 in tracheobronchial lymph nodes (249). PLN could represent another niche beyond the small intestine in which M2 macrophages and antimicrobials have an opportunity to modulate islet autoimmunity, by directly interacting with diabetogenic T cells. Expression of an AMP within the pancreas, including a subset of β-cells, was not expected. Surprisingly, islet-associated CAMP was detected in sterile conditions, including islets at the embryonic stage. The embryonic environment is devoid of microbes and the endocrine pancreas is not inflamed during the pre-natal period, indicating that islet-associated embryonic CAMP was not associated with infection or autoimmunity. In postnatal rats, $CAMP^+$ cells were more frequent in inflamed islets, situated in close proximity to infiltrating $CD68^+$ macrophages. CAMP in inflamed islets could play a counter-inflammatory role during insulitis, analogous to wound healing. CAMP-mediated engagement of EGFR promoted keratinocyte migration, an effect associated with the peptide's wound healing capacity in skin (250). In a tumour study, CAMP was identified as an agonist for the insulin-like growth factor 1 receptor (IGF-1R)(231). This receptor is expressed by β-cells and IGF-1R signalling contributes to islet homeostasis (251-255), reinforcing the possibility that CAMP could stimulate islet growth. Also, IGF-1 itself has been reported to stimulate *Camp*

gene expression (232). Thus, a paracrine mechanism involving IGF-1/IGF-1R signalling and CAMP is possibly active in islets and could be a novel aspect of islet homeostasis.

Inflammation and β-cell regeneration are closely associated (116). During β-cell destruction, the immune system likely interprets insulitis as a wound, with enhanced CAMP production contributing to a repair program. The number of islet-associated $CAMP^+$ cells positively correlated with total islet number among asymptomatic BBdp rats, suggesting an association with β-cell expansion. Also, $CAMP^+$ cells were more frequent in islets from asymptomatic and diabetic BBdp rats compared with control BBc rats, indicating upregulation during inflammation. Examples of inflammatory conditions promoting β-cell expansion include pancreatic duct ligation and partial pancreatectomy (256). Residual β-cells have been found in patients with T1D (257-259). CAMP was present in islets from patients, indicating a role in islet homeostasis even after T1D onset (**Fig. 5.7C**), possibly reflecting an attempt at regeneration. Other findings supportive of CAMP-associated islet growth promotion include its presence in embryonic islets and regenerative tubular complexes as well as upregulation of $insulin^+$ extra-islet clusters (EIC) in CAMP/LL-37-treated BBdp rats.

There is only one publication describing the presence of CAMP in islets and involvement of this peptide in T1D pathogenesis (260). The recent report by Diana *et al*. described a mechanism of innate immune interplay leading to the establishment of insulitis and T1D in NOD mice (260). β-cell apoptosis in young NOD mice resulted in infiltration of "innate-like" $CD5^+$ B-1a lymphocytes that secreted double-stranded self-DNA-binding IgG antibodies (260). CAMP from an islet-infiltrating neutrophil source interacted with the self-

DNA-specific IgG immune complexes and potentiated pro-inflammatory IFN-α secretion from plasmacytoid DC. These early innate inflammatory events were required for accumulation of $CD8^+$ IFN-γ-expressing T cells in PLN (260). Some, but not all aspects of this study were consistent with the present findings. For example, increased islet-associated CAMP was observed during insulitis in BBdp rats, consistent with the Diana *et al*. report. In the present study, inflamed islets from NOD mice displayed CAMP expression in endocrine cells as well as a subset of immune cells included among the immune infiltrate, which could be neutrophils. In the Diana *et al*. report, neutrophils were described as the CAMP-producing population whereas endocrine-based detection was not reported. The role of islet-associated CAMP is a new research area that could lead to new insights into islet homeostasis during development, health, and inflammation. We have additional preliminary data that CAMP affords protection from T1D in BBdp rats. Our group performed a study in which cobalt protoporphyrin (CoPP) was injected into BBdp rats to induce the protective CD163/HO-1 pathway (261), which resulted in lower T1D incidence compared with saline-injected animals (Husseini *et al*., unpublished results). Interestingly, the number of $CAMP^+$ cells and *Camp* gene expression were significantly increased in the jejunum of protected CoPP-treated BBdp rats (Husseini *et al*., unpublished results). In addition, CAMP/LL-37 injections in BBdp rats resulted in an increased number of duct-associated EIC, indicating enhanced islet neogenesis (Wang *et al*., unpublished results).

CONCLUSION

Protective HC feeding increased small intestinal expression of the host defense peptide CAMP. CAMP was enriched in M2 macrophages in immune tissues associated with the gastrointestinal tract. Unexpectedly, CAMP was also observed in islets, including target β-cells. CAMP correlated with total islet number and was increased during insulitis, highlighting a potential novel role in islet homeostasis and T1D pathogenesis. CAMP could function as an islet trophic factor in the endocrine pancreas. Injection of CAMP/LL-37 into young BBdp rats increased the abundance of protective probiotic bacteria and islet neogenesis. Thus, CAMP represents a previously unrecognized innate immune hub linking intestinal defenses, pancreatic immunity, and islet biology.

CHAPTER 6. Pancreatic abnormalities precede insulitis in BBdp rats

ABSTRACT

Background: An increased understanding of the pre-insulitic pancreatic microenvironment could provide novel insights relating to target tissue features contributing to T1D pathogenesis. We previously reported that the pancreas of young BBdp rats displayed increased islet expansion before insulitis, reflected in part by increased cell cycle entry (123). The objective of the present study was to further explore intrinsic target tissue abnormalities.

Methods: Analysis of the G1/S cell cycle inhibitor $p16^{INK4a}$ in β-cells of young BBdp and BBc rats fed cereal or HC was performed using immunohistochemistry, confocal microscopy, and morphometry. Partial (30%) pancreatectomies (PPx) in cereal-fed BBdp rats were performed at 30 d to prospectively biopsy the pancreata; biopsied tissue and RNA were collected for retrospective analysis determined by T1D outcome. Gene expression profiles in retrospectively classified pre-diabetic and resistant pancreata were compared using PCR arrays and microarrays.

Results: β-cells from cereal-fed BBdp rats displayed decreased p16-associated cell cycle inhibition compared with either HC-fed diet-protected BBdp rats or cereal-fed BBc rats. Prospective PPx pancreata of pre-diabetic animals displayed increased endothelial inflammation associated with infiltrating $CD68^+$ macrophages. In parallel, pancreatic gene expression profiling revealed induction of pro-inflammatory factors in conjunction with upregulated regenerative/growth factors in pre-diabetic BBdp rats.

Summary: The pancreata of young BBdp rats were characterized by increased islet proliferative potential and increased inflammatory gene expression preceding islet inflammation.

INTRODUCTION

There is an incomplete understanding of the early stages of T1D pathogenesis in the target pancreas leading to β-cell destruction (262; 263). Pancreatic β-cells represent the primary targets of islet autoimmunity and therefore play a central role in T1D pathogenesis (1). As the decrease in β-cell mass is a gradual destructive process, there is likely an opportunity to target residual cells for expansion (118). There is evidence of residual β-cells, possibly arising by a regenerative attempt, in subjects with recent onset T1D (257; 258) and even in patients with longstanding T1D (259; 264; 265). The most potent methods of inducing β-cell regeneration involve pancreatic injury and usually include an inflammatory component; examples include duct ligation, transgenic cytokine production, and administration of β-cell toxins (256; 266). Enhanced islet regeneration was observed in transgenic mice expressing IFN-γ (267), indicating that pro-inflammatory and pro-apoptotic factors can also drive β-cell proliferation. Accordingly, anti-inflammatory interventions were found to suppress the enhanced β-cell proliferation characteristic of young NOD mice (116; 268). An intriguing possibility is that abnormal islet expansion in early life could represent a diabetes-promoting pancreatic phenomenon in itself.

We previously evaluated islet homeostasis in young BBdp rats and reported that enhanced islet expansion relative to BBc rats occurred before development of insulitis (123). At various points during the pre-insulitic period (14, 23, 30, and 45 d), increased numbers of EIC were observed in the pancreata of young BBdp rats compared with BBc counterparts (123; 269). Also, EIC from BBdp rats displayed increased proliferation on the basis of BrdU incorporation compared with EIC from BBc rats (123). EIC are considered islet precursor

structures that develop into larger islets (123; 269; 270). Consistent with increased numbers of EIC in BBdp rats, islet area fraction of small, medium, and large islets was also increased in BBdp rats during the pre-insulitic period, indicating that the proliferating EIC were likely developing into islets (123). A greater percentage of islet cells from BBdp rats was in the G1 cell cycle phase compared with BBc rats, suggesting increased cell cycle entry and proliferative capacity (123). Thus, altered cell cycle regulation resulting in enhanced expansion of islets was a specific target tissue abnormality in young BBdp rats (123).

The cell cycle inhibitor $p16^{INK4a}$ (p16), a member of the inhibitory kinase (INK) family, plays an age-associated role in limiting β-cell proliferation by potent inhibition of cyclin-dependent kinase 4 (cdk4), resulting in G0/G1 arrest and prevention of G1/S cell cycle progression (271-273). Increased p16 in β-cells of transgenic mice was associated with decreased β-cell mass, impaired glucoregulation, and deficient regenerative capacity (274). In the present *Chapter* (see also (275)), increased proliferative potential was observed in young BBdp islets compared with BBc islets on the basis of decreased p16 expression in β-cells. In addition, prospective gene expression analyses of pancreatic PPx biopsies revealed evidence of increased islet-associated regenerative/growth factors and pro-inflammatory immune factors in the pancreata of pre-diabetic BBdp rats. Endothelium-associated inflammation (perivascular inflammation) was an early lesion in pre-diabetic BBdp rats that preceded insulitis. Overall, enhanced but futile islet proliferative potential in association with pancreatic inflammation was a target tissue abnormality linked to T1D in BBdp rats.

RESEARCH OVERVIEW

Rationale

Exploration of the pre-insulitic pancreata of BBdp rats could provide novel insights relating to target tissue features contributing to T1D pathogenesis. Pancreatic abnormalities in susceptible subjects could attract immune cells during the early stages of T1D development (263). Prospective pancreatic gene expression profiling in young BBdp rats before appearance of classic insulitis could identify factors underlying risk for T1D progression.

Hypotheses

(i) Alterations in islet homeostasis are present in the pancreata of young BBdp rats during the period preceding islet inflammation, which could be influenced by diabetes-modifying diets; *(ii) The pancreata of pre-insulitic BBdp rats that progress to overt T1D are distinct from the pancreata of BBdp counterparts that ultimately remain diabetes-free*.

Research Questions

1. *Are β-cell cycle abnormalities present in young BBdp rats and if so, are these modified by feeding a diabetes-protective HC diet?*

2. *Can pancreas-specific biomarkers of altered T1D risk be identified in young BBdp rats using a partial pancreatectomy-based prospective strategy?*

RESULTS

Increased proliferative potential of BBdp β-cells associated with decreased p16 expression

We previously reported that young BBdp rats displayed increased islet expansion compared with BBc rats during the period preceding islet inflammation, which was associated with an increased proportion of islet cells at the G1 cell cycle phase (123; 269). To determine the basis of altered islet expansion in young BBdp rats, cell cycle inhibition was evaluated in β-cells before insulitis. The cell cycle inhibitor p16 is an important checkpoint regulator of G1/S cell cycle transition (271) that influences β-cell expansion potential (273). In the pancreas of pre-insulitic BBdp rats, p16 expression was detected in a subset of nuclei in islets and acinar tissue; conversely, labeling was not observed in the jejunum (**Fig. 6.1A**), indicating active proliferation in the small intestine relative to the pancreas. A subset of β-cells expressed p16 in large islets of BBdp rats but not insulin$^+$ EIC, structures thought to represent new islet formation (**Fig. 6.1B**). To determine whether alteration of p16 expression was associated with modified islet proliferation indices we previously reported (123), the percentage of p16$^+$ β-cells was quantified at 45 d according to islet size. In cereal-fed BBdp rats, β-cells from small islets had a significantly decreased percentage of p16$^+$ β-cells compared with either medium or large islets (**Fig. 6.1C**). These data are consistent with the increased percentage of BrdU$^+$ β-cells we previously reported in small islets compared with large islets in 30 d BBdp rats (123). In BBc rats, the proportion of p16$^+$ β-cells was similar among small, medium, and large islets (**Fig. 6.1D**), in keeping with our previous report in which proliferation was not influenced by islet size in young BBc rats (123).

FIGURE. 6.1

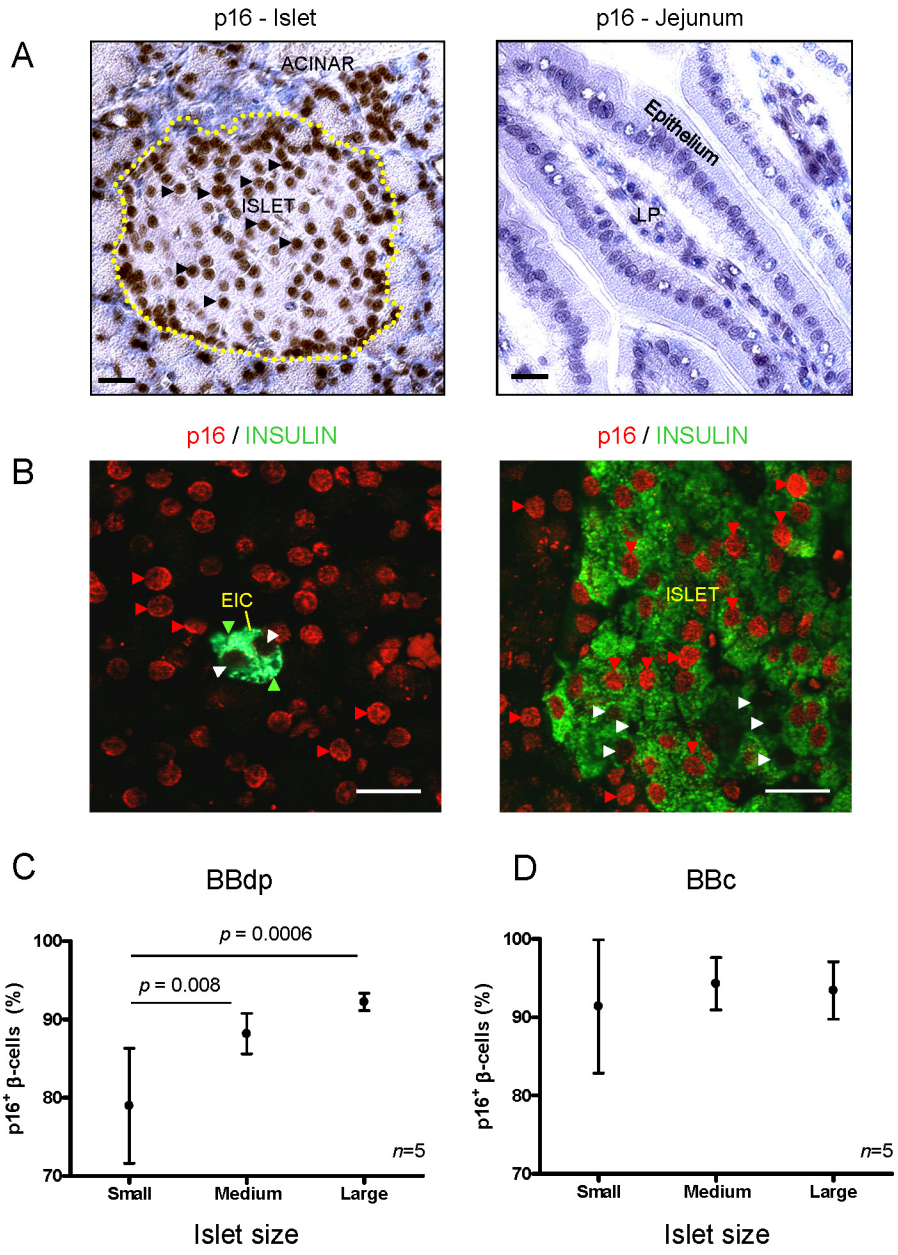

A
p16 - Islet
p16 - Jejunum
ACINAR
ISLET
Epithelium
LP
B
p16 / INSULIN
p16 / INSULIN
EIC
ISLET
C
BBdp
p = 0.0006
p = 0.008
p16+ β-cells (%)
100
90
80
70
n=5
Small
Medium
Large
Islet size
D
BBc
p16+ β-cells (%)
100
90
80
70
n=5
Small
Medium
Large
Islet size

Figure 6.1. Expression of p16 in β-cells increases with islet size in BBdp islets
(**A**) Representative images of p16 staining in pre-insulitic BBdp rat. Pancreatic labeling was observed in a subset of nuclei (left panel) whereas no labeling was detected in the jejunum (right panel); bars=25 μm. (**B**) Immunofluorescence labeling of p16 (red arrowheads) and insulin (green arrowheads) in pancreata of pre-insulitic cereal-fed BBdp rats. Left panel shows an EIC lacking p16 expression (white arrowheads); right panel shows large islet with major subset of nuclei expressing p16; bars = 20 μm. Quantification of $p16^+$ β-cells according to islet size in 45 d cereal-fed (**C**) BBdp and (**D**) BBc rats; small (<2,000 μm^2), medium (2,000-10,000 μm^2), and large (>10,000 μm^2). *n*=5 rats/group. *P*-values obtained using ANOVA and LSD *post-hoc* test. *Note*: Figure modified and reprinted from material published in Wang *et al*., *Journal of Cellular Physiology*, 2010 (275), with permission from John Wiley and Sons.

Taken together, these results established that p16 expression was able to discriminate proliferating and non-proliferating β-cells. Thus, small islets from pre-insulitic BBdp rats displayed decreased cell cycle inhibition whereas BBc islets had equivalent inhibition.

Effects of strain and diet on p16 expression in β-cells

Quantification of $p16^+$ β-cells was performed in 45 d BBdp and BBc rats fed either a cereal diet or HC diet for determination of strain- and diet-associated effects on G1/S cell cycle inhibition in islets of different sizes during the pre-insulitic period. In small- and medium-sized islets, β-cells from cereal-fed BBdp rats had significantly less p16 expression compared with BBc rats (**Figs. 6.2A-B**). Cereal-fed BBdp rats displayed significantly fewer $p16^+$ β-cells in islets of all sizes compared with diet-protected HC-fed rats (**Figs. 6.2A-C**). There were no dietary differences observed among BBc rats, indicating that the dietary effect was specific to the diabetes-prone strain. Thus, islets from 45 d cereal-fed BBdp rats had fewer $p16^+$ β-cells compared with BBc rats or diet-protected BBdp counterparts, suggesting enhancement of proliferative capacity between weaning and insulitis.

Partial pancreatectomy for identification of T1D-predictive features in BBdp pancreata

Prospective characterization of the pre-insulitic pancreas during the pre-diabetic period could reveal critical features of the target tissue microenvironment at an early stage of T1D pathogenesis, which could be central to disease progression. The challenge with studying pre-insulitic time-points as a reflection of true pre-diabetes is that not all BBdp rats progress to overt T1D.

FIGURE. 6.2

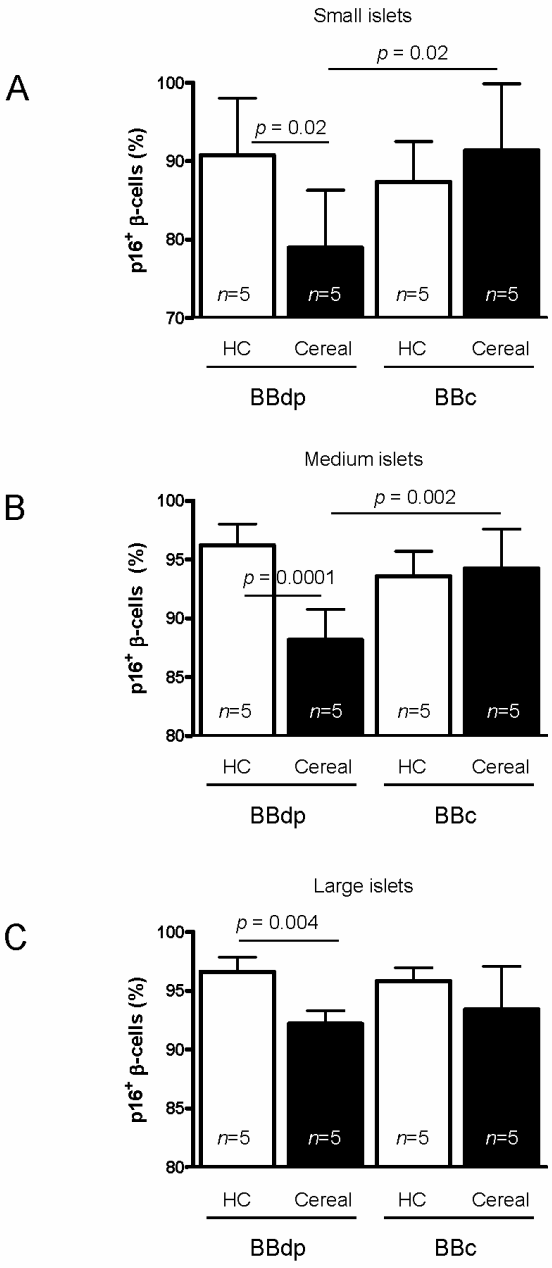

A
Small islets
p = 0.02
p = 0.02
p16+ β-cells (%)
100
90
80
70
n=5
n=5
n=5
n=5
HC
Cereal
HC
Cereal
BBdp
BBc
B
Medium islets
p = 0.002
p = 0.0001
p16+ β-cells (%)
100
95
90
85
80
n=5
n=5
n=5
n=5
HC
Cereal
HC
Cereal
BBdp
BBc
C
Large islets
p = 0.004
p16+ β-cells (%)
100
95
90
85
80
n=5
n=5
n=5
n=5
HC
Cereal
HC
Cereal
BBdp
BBc

Figure 6.2. Decreased p16 expression in β-cells of BBdp rats fed a cereal diet
The number of $p16^{+}$/$insulin^{+}$ cells was quantified in (**A**) small, (**B**) medium, and (**C**) large islets of 45 d BBdp rats and BBc rats fed a cereal diet (filled bars) or HC diet (open bars). *n*=5 rats/group. *P*-values obtained using ANOVA and LSD *post-hoc* test. *Note*: Figure modified and reprinted from material published in Wang *et al*., *Journal of Cellular Physiology*, 2010 (275), with permission from John Wiley and Sons.

Given that target tissue abnormalities were observed in BBdp rats compared with BBc rats before insulitis (123), a prospective study was designed to identify pancreatic markers or features associated with T1D. A partial pancreatectomy (PPx)-based strategy was used to obtain biopsies from pre-insulitic cereal-fed BBdp rats. Such a strategy has never been reported using rodent models of spontaneous T1D. BBdp rats were weaned onto a cereal-based diet at 23 d. Pancreas biopsies were obtained from animals at 30 d, which corresponded to the period following weaning but preceding onset of classic insulitis. By 150 d, rats had either developed T1D or remained asymptomatic. Thus, at 150 d, all 30 day samples could be retrospectively classified in a definitive manner as either "pre-diabetic" or "resistant". Two separate PPx experiments were performed, with tissue from the first study used for immunohistochemistry and morphometry and tissue from the second study used for gene expression screening (**Fig. 6.3A**). In NOD mice, PPx (50%) at 8 weeks of age significantly inhibited T1D development (263). Similarly, 90% PPx before insulitis also afforded protection from T1D (276). Pancreatectomized and sham-operated BBdp rats displayed similar T1D incidence of ~50% (**Fig. 6.3B**). Thus, the 30 d PPx strategy was validated and samples were appropriate for prospective analysis of the target tissue.

Perivascular inflammation is an early inflammatory lesion in pre-diabetic BBdp rats

Obtaining prospective BBdp pancreatic samples provided a unique opportunity to explore the target tissue in susceptible rats that either developed T1D or remained diabetes-free. We previously reported strain- and diet-associated cell cycle abnormalities in BBdp islets (123; 275). Therefore, the cell cycle was similarly evaluated by Proliferating Cell Nuclear Antigen (PCNA) analysis (123; 275) to determine whether islet-associated proliferative imbalances were present in pre-diabetic rats.

FIGURE. 6.3

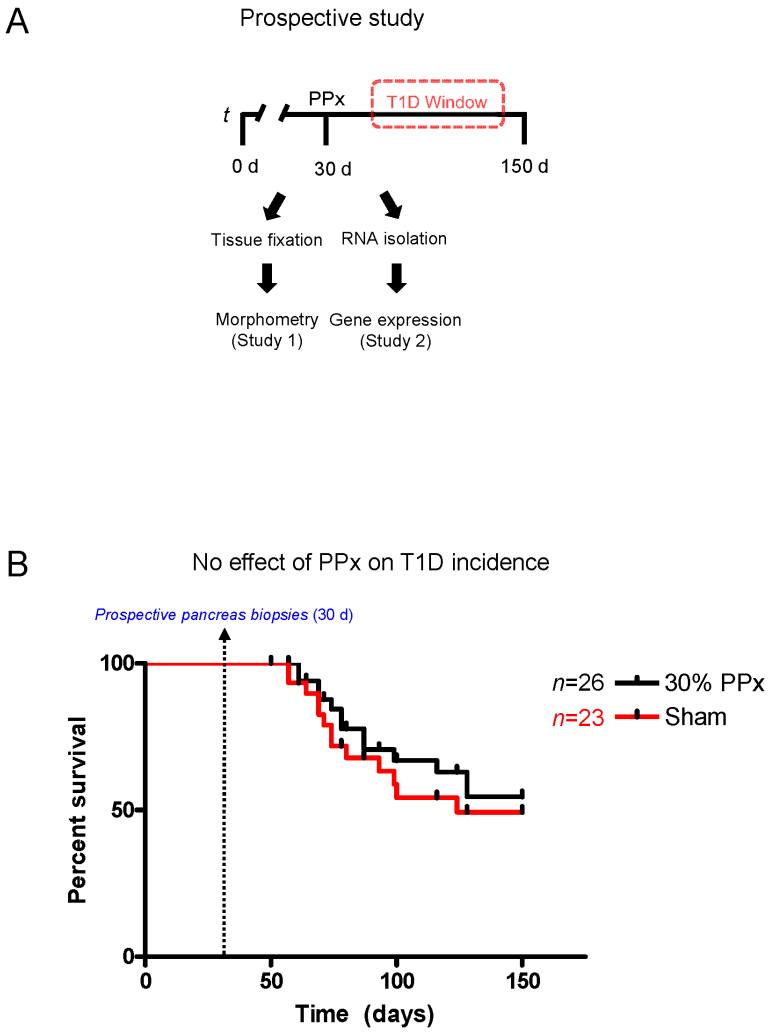

A
Prospective study
t
PPx
T1D Window
0 d
30 d
150 d
Tissue fixation
RNA isolation
Morphometry
(Study 1)
Gene expression
(Study 2)
B
No effect of PPx on T1D incidence
Prospective pancreas biopsies (30 d)
Percent survival
100
50
0
0
50
100
150
Time (days)
n=26
30% PPx
n=23
Sham

Figure 6.3. Partial pancreatectomy for evaluation of pre-diabetic pancreata of BBdp rats

Outline of PPx strategy. (**A**) PPx experiment used to obtain biopsy tissue for immunohistochemistry and morphometry studies as well as RNA for gene expression screening experiments. (**B**) Influence of PPx on survival compared with sham-operated rats; PPx (*n*=26), sham-operated (*n*=23). Kaplan-Meier survival plot indicating the percentage of cereal-fed BBdp rats that remained free of T1D until ~150 d; PPx did not significantly modify T1D incidence compared with sham-operated cereal-fed controls.

The proportions of islet cells distributed at the G0-, G1-, and S-phases of the cell cycle were virtually identical in prospective pre-diabetic and resistant pancreata (**Fig. 6.4A**). Macrophages are among the earliest immune cells to infiltrate islets during T1D development (265). Thus, $CD68^+$ macrophages were quantified in pancreata in association with blood vessels (**Fig. 6.4B**), ducts (**Fig. 6.4C**), and islets (**Fig. 6.4D**). The number of endothelium-associated $CD68^+$ cells was significantly increased in prospective pancreata of pre-diabetic BBdp rats compared with resistant counterparts. No significant differences in duct-associated or islet-associated $CD68^+$ cells were observed in prospective 30 d pancreata. Thus, macrophage-associated perivascular inflammation was an early inflammatory lesion preceding islet inflammation in the pancreas of BBdp rats.

Altered immune gene expression in the pre-diabetic BBdp pancreas precedes insulitis

PCR Array screening was used to identify CAMP as a novel T1D factor in *Chapter 5* (103). A similar screening strategy was used to explore immune-associated gene expression in prospective BBdp pancreata using "Rat Inflammatory Response and Autoimmunity" PCR Arrays (Qiagen/SABiosciences, Mississauga, Canada) that contained primers for a large panel of immune genes. Results are presented on a scatterplot (**Fig. 6.5**), indicating expression values from pooled pre-diabetic pancreatic samples compared with resistant samples (control group). Values ≥ 2 fold identified immune genes upregulated in pre-diabetic rats whereas values ≤ 2 fold identified genes downregulated in pre-diabetic rats. Pre-diabetic pancreata displayed increased transcription of *Lta*, the gene encoding lymphotoxin alpha (LTα). LTα is a pro-inflammatory factor implicated in chronic inflammation and autoimmunity (277).

FIGURE. 6.4

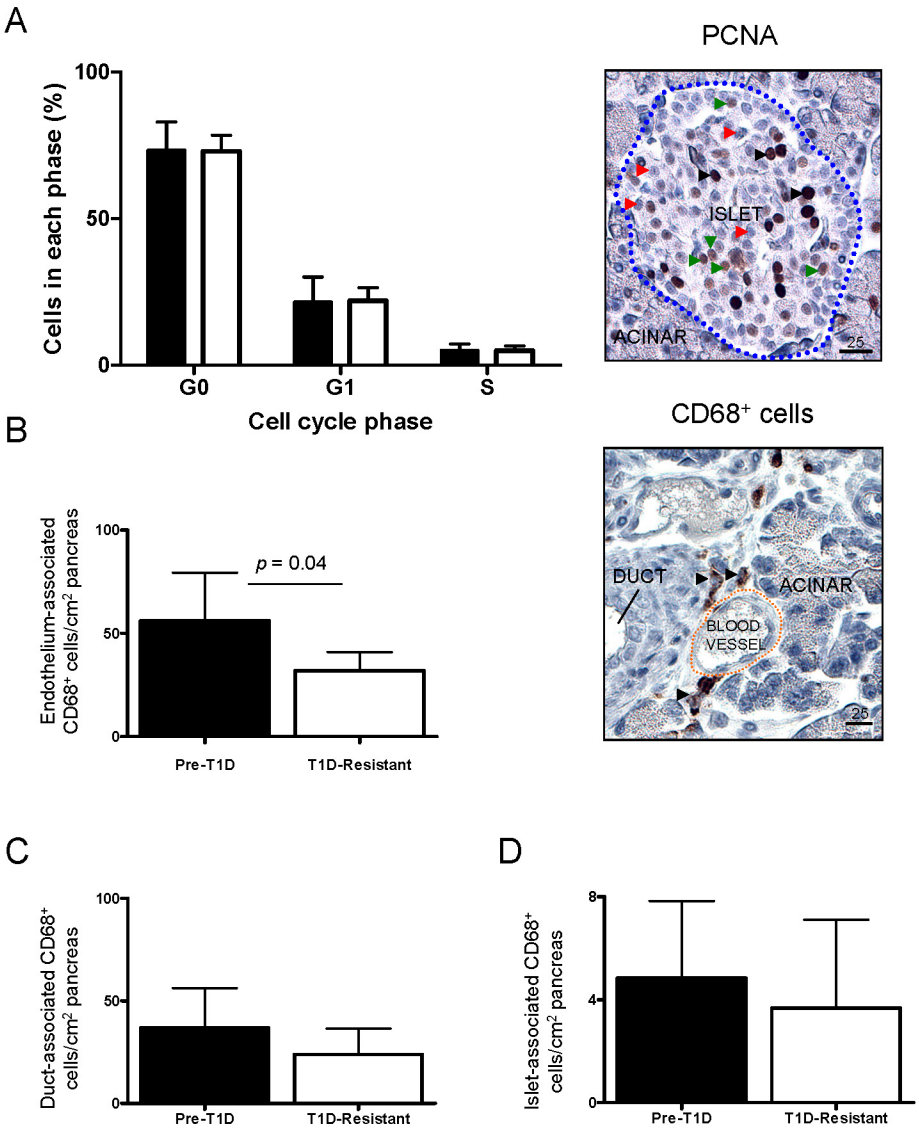

A
PCNA
Cells in each phase (%)
100
50
0
G0
G1
S
Cell cycle phase
ISLET
ACINAR
25
B
CD68+ cells
Endothelium-associated CD68+ cells/cm2 pancreas
p = 0.04
Pre-T1D
T1D-Resistant
DUCT
ACINAR
BLOOD VESSEL
C
Duct-associated CD68+ cells/cm2 pancreas
D
Islet-associated CD68+ cells/cm2 pancreas
8
4

Figure 6.4. Endothelium-associated inflammation in the pre-diabetic BBdp pancreas
(**A**) PCNA-based cell cycle evaluation of G0-, G1-, and S-phase distribution among islet cells of pre-diabetic (filled bars) and resistant (open bars) BBdp rats. Right panel shows representative image of 30 d BBdp islet with nuclei in G0 (red arrowheads), G1 (green arrowheads), and S (black arrowheads); bar=25 μm. (**B**) Quantification of endothelium-associated $CD68^+$ cells in pancreata of pre-diabetic (filled bars) and resistant (open bars) BBdp rats. Right panel shows $CD68^+$ cells (black arrowheads) in vicinity of pancreatic vasculature; bar=25 μm. *P*-value obtained using Student's *t*-test. Quantification of (**C**) duct-associated and (**D**) islet-associated $CD68^+$ cells in prospective pancreata.

FIGURE. 6.5

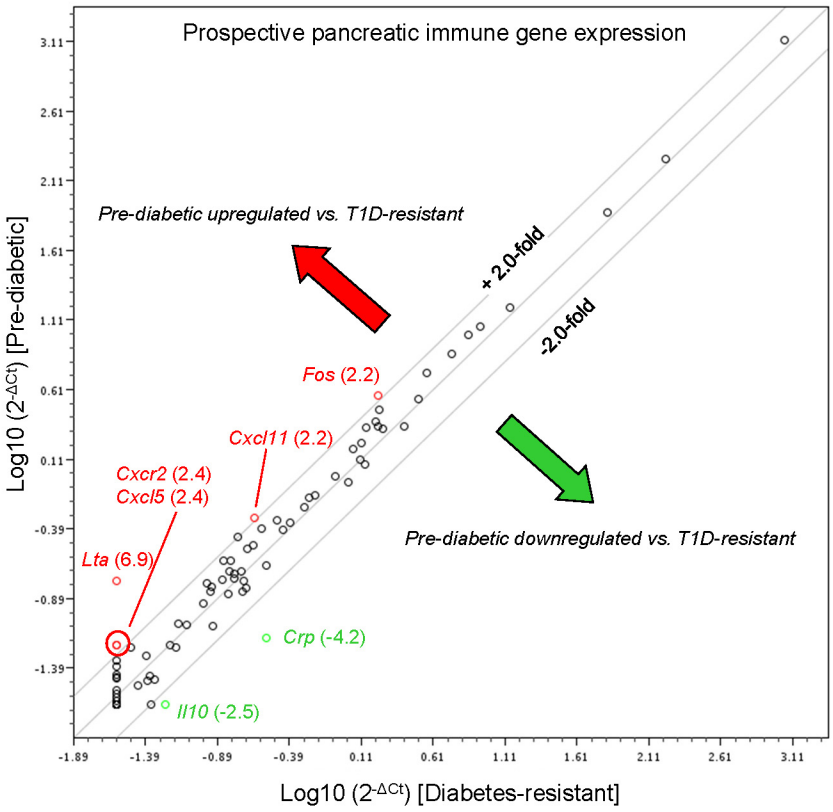

Prospective pancreatic immune gene expression
Pre-diabetic upregulated vs. T1D-resistant
+ 2.0-fold
-2.0-fold
Pre-diabetic downregulated vs. T1D-resistant
Fos (2.2)
Cxcl11 (2.2)
Cxcr2 (2.4)
Cxcl5 (2.4)
Lta (6.9)
Crp (-4.2)
Il10 (-2.5)
Log10 (2-ΔCt) [Pre-diabetic]
Log10 (2-ΔCt) [Diabetes-resistant]
3.11
2.61
2.11
1.61
1.11
0.61
0.11
-0.39
-0.89
-1.39
-1.89

Figure 6.5. Transcriptional profiling of immune genes in prospective pancreata of BBdp rats

PCR arrays for "Rat Inflammatory Response and Autoimmunity" (Qiagen/SABiosciences, Mississauga, Canada) were used to evaluate pancreatic immune gene expression. Scatter plot comparing expression of immune genes in prospective pre-diabetic and resistant pancreata. Genes indicated in red were upregulated ≥ 2 fold in pre-diabetic pancreata; genes indicated in green were downregulated ≥ -2 fold in pre-diabetic pancreata.

In addition, transcription of the chemokine-associated genes *Cxcr2* (CXCR2), *Cxcl5* (CXCL5), and *Cxcl11* (CXCL11) was increased in pancreata from pre-diabetic rats whereas *Crp* (C-reactive protein) and *Il10* (IL-10) were downregulated.

Microarray exploration of prospective pre-diabetic BBdp pancreata

Using pooled samples, another gene expression experiment was performed using microarrays (Affymetrix Rat 1.0 ST microarrays, Affymetrix, Santa Clara, USA) to achieve a global picture of pancreatic transcription in prospective BBdp pancreata. An abbreviated gene list from the microarray experiment displaying the top upregulated genes in pre-diabetic vs. resistant pancreata is presented in **Fig. 6.6**. Candidates of interest are briefly described below.

Trim26: *Trim26* was the most upregulated gene in the pre-diabetic BBdp pancreas. The tripartite motif-containing (TRIM) protein family contains multifunctional E3 ligases with anti-viral activity and inflammatory function (278). It is being increasingly recognized that alteration of various TRIM family members could contribute to autoimmunity and inflammatory diseases (278). There are currently no reports linking TRIM26 and T1D, nor any description of this factor in the pancreas.

Reg3 family: The *Reg3a* (Reg3α) and *Pap* (Reg3β) genes were upregulated in pre-diabetic BBdp pancreata. Interestingly, when short-term injections of either INGAP-P (another Reg3 family member (279)) or human Reg3α/β were administered to normal mice, a 1.5-2-fold increase in the cell mass of EIC was observed (280).

FIGURE. 6.6

Experiment 118F: 30 d Pancreas comparison - Pre-diabetic vs Resistant BBdp rats
Affymetrix Rat 1.0 GENE ST microarrays

	Symbol	Description	Fold change (Pre-diabetic vs Resistant)
	Trim26	Tripartite motif-containing protein 26 (Zinc finger protein 173). [S	9.76
	Ngfb	Beta-nerve growth factor precursor (Beta-NGF). [Source:UniProt	5.03
	Hdac1_predicted	Histone deacetylase 1 (HD1). [Source:UniProtKB/Swiss-Prot;Acc	3.91
	Robo4	Roundabout homolog 4 precursor. [Source:UniProtKB/Swiss-Pro	3.87
	NP_001099949.1	chloride channel 6 [Source:RefSeq_peptide;Acc:NP_001099949	3.76
*	Reg3a	Regenerating islet-derived protein 3 alpha precursor (Reg III-alpl	3.76
	Serpinb6b	serine (or cysteine) proteinase inhibitor, clade B, member 6b [Sc	3.49
	Cgm3	carcinoembryonic antigen gene family (CGM3) [Source:RefSeq_	3.47
	Slc6a13	Sodium- and chloride-dependent GABA transporter 2. [Source:U	3.34
	Adcy4	Adenylate cyclase type 4 (EC 4.6.1.1) (Adenylate cyclase type IV	3.26
	NP_001100993.1	chromodomain helicase DNA binding protein 2 [Source:RefSeq_	3.14
*	Pap	Regenerating islet-derived protein 3 beta precursor (Reg III-beta)	3.14
	RGD1561089_predicted	late cornified envelope 1F [Source:RefSeq_peptide;Acc:NP_001	3.00
	NP_001099720.1	sialic acid binding Ig-like lectin 10 [Source:RefSeq_peptide;Acc:	2.98
	Slc26a5	Prestin (Solute carrier family 26 member 5). [Source:UniProtKB/S	2.98
	MGC94199	Uncharacterized protein C8orf37 homolog. [Source:UniProtKB/S	2.84
	Zfp707	zinc finger protein 707 [Source:RefSeq peptide;Acc:NP_001074	2.78
	Cbwd1	COBW domain-containing protein 1 (Cobalamin synthetase W do	2.78
	Egr1	Early growth response protein 1 (EGR-1) (Protein Krox-24) (Tran	2.77
	NP_001101769.1	ets variant gene 4 (E1A enhancer binding protein, E1AF) [Sourc	2.76
	Gp49b	glycoprotein 49b [Source:RefSeq_peptide;Acc:NP_001013916]	2.73
	NP_001103001.1	similar to expressed sequence C79407 (LOC689296), mRNA [Sc	2.70
	ISK7_RAT	Serine protease inhibitor Kazal-type 7 precursor (Esophagus can	2.70
	Mlph	melanophilin [Source:RefSeq_peptide;Acc:NP_001012135]	2.69
	Tesb	hypothetical protein LOC407788 [Source:RefSeq peptide;Acc:NI	2.69
	LOC503419	similar to Expressed sequence AW146242 (predicted) (RGD130	2.68
	Ccdc32	Protein CCDC32. [Source:UniProtKB/Swiss-Prot;Acc:Q561K4]	2.63
	Ly49i3	immunoreceptor Ly49i3 [Source:RefSeq_peptide;Acc:NP_0010	2.62
	Sh2d2a	SH2 domain protein 2A [Source:RefSeq_peptide;Acc:NP_99748	2.58
	Dgkg	Diacylglycerol kinase gamma (EC 2.7.1.107) (Diglyceride kinase	2.55
	U6	U6 spliceosomal RNA [Source: RFAM 9.0]	2.54
	Opn4	Melanopsin (Opsin-4). [Source:UniProtKB/Swiss-Prot;Acc:Q8R4	2.52
	Cdk2	Cell division protein kinase 2 (EC 2.7.11.22). [Source:UniProtKB/	2.52
	Kcnab2	Voltage-gated potassium channel subunit beta-2 (K(+) channel s	2.51
	Palb2	Partner and localizer of BRCA2 [Source:UniProtKB/Swiss-Prot;A	2.50
	Tg	Thyroglobulin precursor. [Source:UniProtKB/Swiss-Prot;Acc:P06	2.50
	Arhgap20	Rho GTPase-activating protein 20 (Rho-type GTPase-activating p	2.50
	NP_001100600.1	F-box only protein 24 [Source:RefSeq_peptide;Acc:NP_001100	2.41
	Ndnl2	Ndnl2 protein (Fragment). [Source:UniProtKB/TrEMBL;Acc:Q4KM	2.40
	PTRF_RAT	Polymerase I and transcript release factor (Calvin) (cav-p60). [Sc	2.38
	Olr1654_predicted	olfactory receptor Olr1654 [Source:RefSeq_peptide;Acc:NP_068	2.37
	Lgi1	Leucine-rich glioma-inactivated protein 1 precursor. [Source:UniP	2.34
	Cntnap2	Contactin-associated protein-like 2 Precursor (Cell recognition m	2.33

Figure 6.6. Microarray screening of pancreatic gene expression in prospective pancreata of BBdp rats

Microarrays (Affymetrix Rat 1.0 ST microarrays, Affymetrix, Santa Clara, USA) were used to compare transcription in prospective pancreata. A partial gene list is shown of the top upregulated factors in the pre-diabetic BBdp rats. The gene list was obtained using AltAnalyze software (http://www.altanalyze.org; Gladstone Institutes, UCSF). Upregulated *Reg3a* and *Pap* (*Reg3b*) genes in the pancreata of pre-diabetic BBdp rats are highlighted.

These findings are in keeping with our group's previous findings of increased duct-associated insulin$^+$ EIC in young BBdp rats injected with INGAP-P (275). Inflammatory cytokines promote endogenous expression of Reg proteins, suggesting they are activated during inflammation and contribute to islet regeneration (281). Upregulation of *Reg3a* and *Reg3b* in the pre-insulitic pancreata of pre-diabetic BBdp rats is consistent with the notion that abnormally increased islet expansion is associated with promotion of T1D (123).

SUMMARY

β-cells from young BBdp rats displayed decreased p16-associated cell cycle inhibition compared with BBc rats, suggesting increased proliferative potential; this difference was not seen in BBdp rats fed a diabetes-protective HC diet. PPx at 30 d was validated as a strategy for prospective pancreatic analysis for identification of T1D-predictive factors. Perivascular inflammation was an early feature distinguishing pre-diabetic BBdp rats from resistant BBdp rats. In addition, pre-diabetic pancreata displayed increased pro-inflammatory immune gene expression compared with resistant counterparts. Microarray exploration revealed enrichment of multiple regenerative factors in pre-diabetic rats, indicating increased islet expansion potential preceding insulitis and T1D progression. Taken together, these findings lead one to speculate that abnormally increased islet expansion occurring in the pre-insulitic period could potentiate insulitis and T1D.

DISCUSSION

Pancreatic abnormalities inherent to the target tissue possibly underlie T1D susceptibility and could promote initial immune cell infiltration. The target tissue was evaluated in young BBdp rats to further explore the basis for the futile upregulation of islet expansion we

previously reported in these animals that occurred before islet inflammation (123). In β-cells from young BBdp rats, decreased p16-associated cell cycle inhibition was observed compared with BBc counterparts, in keeping with our previous studies describing increased proliferation in the diabetes-prone strain (123; 269). We previously reported that an increased proportion of islet cells from young BBdp rats were in the G1-phase of the cell cycle compared with BBc rats (123). In the present study, decreased p16 expression was observed in β-cells from BBdp rats compared with BBc rats. Taken together, these findings are indicative of abnormally increased proliferation potential associated with T1D susceptibility in BBdp rats. Thus, the increased potential for islet expansion during the pre-insulitic period represents a pancreatic abnormality in BBdp rats that could be essential for T1D progression (123). Despite the increased islet expansion potential in young BBdp rats, β-cell mass was not increased compared with BBc rats at either 30 d or 45 d (123). Similar results have been reported in NOD mice; despite decreased β-cell mass, 8 week-old NOD mice displayed increased β-cell proliferation compared with NOD/*scid* controls prior to T1D onset (122). There was also a greater proportion of small islets in the early postnatal period (1 d) in NOD and NOD/*scid* mice compared with C57BL/6 controls (282). Similarly, we reported an increased number of insulin$^+$ EIC and greater small islet area fraction in young BBdp rats compared with BBc rats (123). We concluded that the induction of islet expansion in young BBdp rats was futile since β-cell mass was not different compared with BBc rats (123). The cereal-associated downregulation of β-cell-specific p16 expression was also futile, as we reported that cereal-fed BBdp rats had lower β-cell mass at 45 d compared with HC-fed animals (275). Thus, there was a nonproductive upregulation of β-cell expansion capacity in young diabetes-prone rodents that did not result in increased β-cell mass. Early

abnormalities involving islet homeostasis and β-cell cycle regulation could represent critical aspects of T1D pathogenesis.

Interestingly, neonatal β-cell apoptosis is a feature of NOD mice and BBdp rats (283), which could provide a physiological basis for enhanced proliferation in young diabetes-prone rodents. In a recent study, injection of a pan-caspase inhibitor in neonatal NOD mice suppressed apoptosis, prevented insulitis, and inhibited T1D (260). In addition, impaired removal of apoptotic cells by macrophages is a feature of NOD mice, with an abnormal load of apoptotic debris playing a role in initiation of an inflammatory immune response (284; 285). Similarly, macrophages from neonatal BBdp rats displayed decreased phagocytosis of apoptotic β-cell debris compared with macrophages from control rats (286), suggesting that abnormalities in apoptosis and macrophage function are important features of T1D initiation in the pancreas of young rodents. However, in our previous study of pre-insulitic BBdp rats, apoptosis was rarely observed at 14 d and 45 d (123). Nevertheless, increased apoptosis could be the basis for enhanced postnatal β-cell proliferation but earlier time points would need to be analyzed. Physiological β-cell apoptosis was described during normal perinatal islet development in humans (287), providing a potential inflammatory stimulus in a diabetes-prone genetic setting with concomitant immune system abnormalities.

A correlation between β-cell proliferation and inflammation is recognized in diabetes-prone rodents but is attributed to islet-infiltrating immune cells providing an expansion stimulus (115). Lower expression of p16 in β-cells of cereal-fed BBdp rats could represent an abnormality contributing to early islet hyperproliferation and immune cell

infiltration. In keeping with this view, neogenic tubular complexes (227), which contain insulin$^+$ cells lacking nuclear expression of p16 (not shown), were infiltrated by CD3$^+$ T cells and CD68$^+$ macrophages ((227) and Wang G.-S. *et al*., unpublished results). In the present study, p16 expression in β-cells was lowest in cereal-fed BBdp rats compared with either cereal-fed BBc rats or diet-protected HC-fed BBdp rats, suggesting that decreased cell cycle inhibition coupled with enhanced proliferative capacity during the pre-insulitic period was associated with increased T1D risk.

Development of a PPx strategy using young BBdp rats afforded the unique ability to prospectively explore the target tissue of pre-diabetic animals. Cell cycle distribution was not different in pre-diabetic or resistant islets at 30 d, indicating that modification of islet proliferative potential was not associated with T1D progression among susceptible rats at this age. This finding is in keeping with our previous report, in which the BrdU index was not different in islets from BBdp rats compared with BBc rats at 30 d (123). Inflammation was next assessed. An increased number of endothelium-associated CD68$^+$ macrophages was observed in pre-diabetic pancreata. No differences in duct-associated or islet-associated inflammation were observed. Thus, perivascular inflammation was the only lesion in the BBdp rat pancreas that distinguished pre-diabetic rats from resistant counterparts at 30 d. Pancreatic perivascular inflammation has been observed in mice at various ages, notably at younger time points in association with pancreatic remodeling (288). Pancreatic infiltration by CD68$^+$ macrophages via the leaky vasculature could lead to establishment of a pro-inflammatory environment in the target tissue before insulitis; Homo-Delarche and Drexhage have proposed a similar scenario underlying early pancreatic inflammation (288). Interestingly, anti-endothelium antibodies were observed in BB-DP rats preceding T1D and

purified antibodies from these rats induced pancreatic vascular leakage when injected into mice (289). Additional papers have also reported increased leakage of pancreatic vasculature in BB-DP rats (290; 291). In a recent human study, peripheral neutrophil deficiency was reported in patients during the period preceding T1D and infiltration of neutrophils was observed in the vicinity of blood vessels in the exocrine pancreas of T1D patients (292). Thus, perivascular inflammation is an early pre-insulitic pancreatic lesion in T1D pathogenesis in rodents and humans. During pancreatic remodeling processes occurring early in life, increased macrophages persist in the target tissue of diabetes-prone rats (293). Tissue resident macrophages can produce cytokines and growth factors, capable of influencing pancreas development and islet homeostasis (288). Determining the phenotype of pancreatic macrophages will be essential in determining their potential effects on pancreas biology and T1D. Preliminary data from our group indicate that injection of the HO-1 inducer cobalt protoporphyrin (CoPP) into BBdp rats inhibited T1D and was associated with pancreatic enrichment of M2-like macrophages co-expressing HO-1, CD68, and CD163 (Husseini *et al*., unpublished results).

PCR array screening of immune factors in prospective pancreata revealed candidate factors implicated in early pancreatic inflammation. Pre-diabetic BBdp pancreatic biopsies displayed an increased expression of the *Lta* gene encoding lymphotoxin alpha (LTα), which is a pro-inflammatory factor implicated in chronic inflammation and autoimmunity (277). Transgenic mice producing LTα from the insulin promoter exhibited early signs of peri-insulitis and insulitis (294), consistent with its induction in pre-diabetic BBdp pancreatic tissue. Various chemokine-associated genes, including *Cxcr2*, *Cxcl5*, and *Cxcl11* were

upregulated in pre-diabetic pancreata and could promote early immune cell attraction. CXCL11 is chemotactic for activated T cells and has been implicated in T_H1-biased inflammation (295). In a study of human pancreatic islets, CXCL11 was induced in the presence of the pro-inflammatory T_H1 cytokine IFN-γ (296). Interestingly, mice lacking the CXCL11 receptor CXCR3 had delayed T1D onset due to inhibition of T cell infiltration into islets (297). Thus, enhanced pancreatic production of CXCL11 (**Fig. 6.5**) could directly contribute to T cell infiltration. CXCL9 and CXCL12 were increased in pancreatic lysates from 4-week old NOD mice compared with either C57BL/6 controls and non-obese diabetes-resistant (NOR) mice (298), consistent with increased pancreatic chemokine production preceding islet inflammation. As with CXCL11, the receptor for CXCL9 is also CXCR3 (298). The CXCR2 receptor has been implicated in islet damage and its inhibition with the drug reparixin, a CXCR1/2 antagonist, was recently demonstrated to improve several aspects of islet transplant outcome in a phase 2 clinical trial (299). In a prospective human study, the plasma concentration of CXCL5 was increased in children with islet autoimmunity (autoantibody-positive) compared with persistent autoantibody-negative subjects (300). Thus, some of these upregulated genes in the pre-diabetic pancreatic microenvironment could be implicated in initiating early inflammation in the BBdp pancreas leading to immune cell recruitment and insulitis. In parallel, the downregulated genes could also confer risk for autoimmunity. C-reactive protein (*Crp*), the most downregulated gene in pre-diabetic pancreata, is a host defense factor with antimicrobial and anti-inflammatory properties that is implicated in removal of apoptotic debris and protection from autoimmunity (301-304). *Il10* was also downregulated in pre-diabetic rats. In a recent study, tolerogenic $CD103^+$ DC in the pancreata of young NOD mice expressed lower *Il10* compared with controls (305). Altered

immune gene expression, characterized by increased inflammatory factors in conjunction with decreased protective factors, preceded insulitis in the pancreas of BBdp rats.

Microarray-based analysis also revealed intriguing features of the pre-diabetic BBdp pancreas. Two genes from the Reg3 family encoding Reg3α and Reg3β were upregulated in pre-diabetic pancreata at 30 d. The link between Reg factors and islet expansion has been recognized for over twenty years (306-308). For example, when isolated islets were exposed to various growth factors, islet cell replication was stimulated in association with increased *Reg* gene expression (306). Reg3 family members are proliferative factors associated with islet expansion (275; 279-281). In a study by De Leon *et al*., PPx induced expression of *Reg3b* in acinar tissue from the pancreatic remnant (309). In the present study, the biopsied tissue was analyzed and not the remnant; in addition, the variable in our experiment was T1D outcome, not surgery. Therefore, it was surprising to observe increased expression of these regenerative factors in young pre-diabetic rats when they are usually induced only upon major injury. However, since enhanced transcription of inflammatory mediators and perivascular inflammation were also observed in pre-diabetic rats, this inflammation could have been sufficient to drive the expression of regenerative Reg3 factors. Upregulation of *Reg* genes in pre-diabetic BBdp pancreata is consistent with the notion that abnormally increased islet expansion is associated with promotion of T1D development (123).

CONCLUSION

The pre-insulitic pancreatic microenvironment is complex and an increased understanding using gene expression screening could help elucidate novel aspects of T1D pathogenesis. The pancreas of pre-diabetic BBdp rats was characterized by increased inflammatory gene expression in parallel with macrophage-associated perivascular inflammation. The PPx-based prospective study was an approach that enabled the discovery of novel target tissue features predictive of T1D. In addition, the induction of regenerative factors in pre-diabetic rats supports the view that increased islet expansion was a pre-insulitic diabetes-promoting feature in BBdp rats.

CHAPTER 7. General Discussion

Summary: T1D is a complex chronic disease influenced by environment and modulated by immune aspects of the gastrointestinal tract (9; 310). The research described in this thesis approached the problem of T1D from an integrative perspective, focusing on several aspects of pathogenesis and attempting to uncover novel links among various contributing factors. The immune phenotype of the jejunum was investigated in detail using two different rat models of spontaneous T1D, BBdp rats (*Chapter 3*) and LEW-DP rats (*Chapter 4*) to determine the association between environment (diet and/or microbes), small intestinal inflammation, and T1D development. Increased T cell infiltration, T_H1-biased gene expression, and deficits in regulatory immune capacity, including $CD163^+$ M2 macrophages, characterized the jejunum of cereal-fed diabetes-prone rats. Importantly, these immune features in the jejunum paralleled risk for T1D development. In *Chapter 5*, CAMP was identified as a novel HC-upregulated antimicrobial factor in the jejunum of diet-protected BBdp rats and was enriched specifically in anti-inflammatory $CD163^+$ M2 macrophages. Unexpectedly, CAMP was also discovered in PLN as well as pancreatic islets, revealing an unrecognized association with immune and endocrine aspects of the target tissue. In islets, CAMP was associated with islet expansion and was modified by islet inflammatory status, indicating novel links with islet homeostasis and T1D pathogenesis. Exogenous CAMP/LL-37 injections in young BBdp rats increased the abundance of diabetes-protective probiotic bacteria and stimulated islet neogenesis, illustrating the multifunctional capacity of this peptide in T1D modulation. In *Chapter 6*, the target tissue was investigated in young BBdp rats to study inherent pancreatic abnormalities implicated in T1D development. In β-cells from pre-insulitic BBdp rats fed a diabetes-promoting cereal diet, there was decreased nuclear expression of the cell cycle inhibitor $p16^{INK4a}$, indicating increased but futile

proliferative potential compared with BBc counterparts that was corrected by protective HC feeding. This suggested that protection was associated with decreased islet proliferation potential. This view was supported and extended by findings from our unique PPx-based prospective screening study, which revealed that expression of islet-regenerative *Reg3* genes was upregulated in pancreata of *bona fide* pre-diabetic rats. These findings confirm and expand on our previous report describing increased islet growth in young BBdp rats compared with BBc rats (123) and support a model in which abnormal islet expansion in the pre-insulitic period promotes T1D.

Cereal antigens promote gut inflammation in diabetes-prone rats: The gastrointestinal tract is the organ that first encounters environmental factors. A major challenge is that environmental factors that influence pathogenesis exert their effects long before the manifestation of islet autoimmunity and hyperglycemia (1). This challenge was circumvented in part by focusing on the gastrointestinal tract, the principal site where environment and the immune system interact and potentially initiate early inflammatory events leading to T1D. When diet and microbes were separated (*Chapter 3*), exposure to cereal antigens was the major environmental inducer of T1D in BBdp rats (103); cereal antigens also promoted T1D in LEW-DP rats (*Chapter 4*). In these strains, cereal feeding promoted jejunal T cell infiltration and pro-inflammatory *Ifng*-associated T_H1 gene expression bias in conjunction with deficits in anti-inflammatory immune cells, indicating common gut immune impairments in two separate rat models of T1D associated with cereal exposure. IFN-γ has consistently been shown to play a major role in stimulating T1D (36; 118; 311-313). IFN-γ can directly impair epithelial barrier integrity by disturbing tight

junction organization (154; 164-166). In BBdp rats, small intestinal inflammation could precede increased gut permeability in the sequence of pathological events leading to T1D, as leakiness appears relatively late (51; 58). This view is supported by a recent study in NOD mice in which small intestinal inflammation developed prior to gut leakiness (314). An intriguing proposition is that the primary intestinal defect in diabetes-prone subjects is inflammation, with increased gut leakiness representing a secondary consequence of this lesion; we previously outlined this possibility in the review by Sonier *et al*. (148). Thus, maintenance of an anti-inflammatory intestinal microenvironment by a low-antigen diet could afford protection from T1D by inhibiting gut leakiness, suppressing chronic intestinal inflammation in response to environmental factors and preventing activation of gut-originating β-cell-reactive immune cells.

Diabetes-protective HC feeding increases small intestinal anti-inflammatory immune cells: In diet-protected BBdp rats, increased $CD163^+$ cells and upregulated *Cd163* gene expression occurred in parallel with a decreased *Ifng*/*Il4* cytokine ratio, indicative of a shift towards a T_H2-biased immune phenotype. The jejunal cytokine milieu could exert a critical influence on M2 macrophage development, as the microenvironment controls macrophage polarization by induction of transcriptional programs (108). In rats, CD163 expression was stimulated by T_H2 cytokine IL-4 (109). Similarly in humans, *CD163* transcription and M2 polarization were driven by immunomodulatory IL-4 and IL-10 signalling (198; 315; 316). LEW-DP rats displayed deficits in both $CD163^+$ M2 macrophages and $Foxp3^+$ T_{reg} compared with LEW-C counterparts. The development and function of $Foxp3^+$ T_{reg} is promoted by M2 macrophages (105). Thus, maintenance of an anti-inflammatory state involves additive interplay among various tolerogenic subsets. There are many reports that impaired adaptive immune

regulation promotes autoimmunity (94; 100; 317-321). Young LEW-DP rats displayed a deficit in jejunal Foxp3$^+$ T_{reg} compared with LEW-C rats, which was corrected by weaning onto a diabetes-protective HC diet. Similarly, an increased proportion of Foxp3$^+$ T_{reg} was observed in HC-fed BBdp rats, but only under sterile conditions. Increased adaptive immune regulation by Foxp3$^+$ T_{reg} could not explain the HC-mediated dietary protection under standard microbe-exposed conditions. Therefore, increased jejunal adaptive immune regulation was not a mechanism of dietary protection in SPF-housed BBdp rats, in contrast to LEW-DP rats. Interestingly, there is evidence that T_{reg} are unstable in various animal and human T1D studies (1). Foxp3$^+$ T_{reg} have been reported to convert to "exT_{reg}" cells that produce IFN-γ, thereby contributing to inflammation and T1D (193; 194). Similarly in humans, increased FOXP3$^+$IFN-γ$^+$ cells were observed in peripheral blood of patients compared with controls (147).

An interesting finding in the study by Parsa *et al*. was that M2 macrophages retained their suppressive phenotype when exposed to pro-inflammatory stimuli (102). Thus, promotion of M2 macrophage expansion in the gut by weaning onto a low-antigen HC diet has the potential to provide persistent anti-inflammatory capacity in the small intestine. Although the work in *Chapter 3* demonstrated that microbes were not required for T1D development (103), microbes could nonetheless represent important environmental T1D modifiers. Interestingly, HC-associated upregulation of CD163$^+$ cells and *Cd163* expression required the presence of commensal microbes, indicating a microbial role in dietary expansion of intestinal M2 macrophages. Microbial metabolites, specifically commensal bacteria-derived short-chain fatty acids, were recently reported in *Science* to promote the

development and function of colonic T_{reg} (322). Similarly, microbes promoted expansion of $CD163^+$ M2 macrophages when these were the main environmental antigens, consistent with a microbial role in M2 macrophage homeostasis.

Protective diet upregulates Camp in the small intestine of BBdp rats: In *Chapter 5*, immune gene expression screening revealed that protective HC feeding induced jejunal *Camp* expression in BBdp rats. Intestinal CAMP – or its modification by diet – has not been previously described in T1D. Interestingly, intestinal CAMP was enriched specifically in $CD163^+$ cells, another novel finding that links this peptide to M2 macrophages. In many colitis studies, CAMP expression was associated with inhibition of gut inflammation (245; 246; 323; 324). In a recent study by our group, anti-inflammatory HO-1 induction via CoPP injections inhibited T1D development in BBdp rats in association with induction of *Camp* expression in the jejunum (Husseini *et al*., unpublished). Decreased jejunal CAMP could enable establishment of a low-grade inflammation due to chronic host contact with bacteria; this could sensitize the host and potentiate aberrant pro-inflammatory immune responses upon cereal exposure. There is accumulating evidence that CAMP is associated with infant protection during early life, suggesting potential involvement in human T1D protection. While studying the immune effects of human breast milk, Cederland *et al*. reported that a combination of lactose and butyrate synergistically upregulated *CAMP* expression in colonic epithelium, monocytes, and macrophages (236), underscoring the importance of this peptide in intestinal homeostasis. Thus, CAMP can be induced by nutritional signals of non-microbial origin, in keeping with HC-associated upregulation described in *Chapter 5* (103).

CAMP was also identified as a component of both amniotic fluid and breast milk, suggesting its involvement in fetal and infant protection, respectively (215). Prolonged

breastfeeding was associated with decreased islet autoimmunity in susceptible children and gut microbes of breastfed infants displayed a predominance of probiotic *Bifidobacteria* upon early bacterial colonization during the first 20 days of life compared with formula-fed children (325; 326). Early nutritional status has a dramatic effect on initial shaping of commensal microbes (325) and AMPs in breastmilk could be critical to this process. Thus, nutritional status can have a direct influence on AMP production, with subsequent effects on the composition of the microbiota. In our pilot study, injections of CAMP/LL-37 in young BBdp rats enhanced the abundance of *Bifidobacteria*, demonstrating a novel method of intestinal probiotic enrichment that could inhibit T1D, as demonstrated by other probiotic studies in diabetes-prone models (88; 211). This experiment represented the first attempt to administer exogenous CAMP/LL-37 in a T1D model and the effects are consistent with the protection afforded by breastfeeding.

Discovery of CAMP in the target pancreas: Detection of endogenous CAMP in PLN and especially in pancreatic islets was unexpected. PLN-associated CAMP was enriched in $CD163^{+}$ macrophages, as in the jejunum. Thus, the immunomodulatory effects of CAMP could partly contribute to M2 macrophage-mediated protection from T1D (102), by maintaining anti-inflammatory states in the jejunum and PLN, both critical immune organs modulating T1D development (61). There is some evidence that islet cells express various innate immune proteins, which is intriguing since they are endocrine cells. Specifically, rodent and human islets have been reported to express innate immune receptors including CD14, TLR2, TLR3, TLR4, and TLR9 (116; 327; 328), although their role in the endocrine pancreas remains unclear. In a recent report by Lee *et al*. in *Cell*, the chemokine receptor CX3CR1 was unexpectedly observed in islets and found to be essential for islet homeostasis

(329). Specifically, islet interaction between CX3CR1 and its chemokine ligand CX3CL1 were required for insulin secretion and maintenance of the differentiated β-cell phenotype (329). Thus, innate immune signalling is emerging as a previously under-recognized regulator of β-cell function. CAMP can be added to this growing list of innate immune factors expressed in islets and represents a new player in endocrine pancreas homeostasis. Intriguingly, islet-associated CAMP co-localized with insulin, indicating presence in some β-cells, especially in non-infiltrated islets. Among asymptomatic BBdp rats, there was a positive correlation between islet-associated CAMP expression and total islet number. Among adult animals, islet-associated $CAMP^+$ cells were more frequent in either overt diabetic or asymptomatic BBdp rats compared with BBc rats, indicating enrichment during islet inflammation. Insulitis is potentially interpreted as a wound and CAMP possibly fulfills an analogous repair role. For example, increased CAMP is generated in skin wounds during the resolution phase of inflammation and is thought to contribute to tissue repair (216; 228). Interestingly, CAMP was reported to inhibit pro-inflammatory M1 macrophage responses by inhibiting production of TNF-α and NO, demonstrating strong immunomodulatory capacity (330); M1 macrophages are important pro-inflammatory participants in insulitis (331). Thus, CAMP in islets could promote normal islet growth and repair while also providing immunomodulatory cytoprotection during insulitis.

The ductal epithelium in the pancreas is a potential source of β-cell progenitors during the postnatal period (266). In the present study, rare $CAMP^+$ cells could be detected within the duct-associated epithelium of adult BBdp rats (not shown), possibly representing a postnatal islet progenitor population. CAMP production in embryonic islets and neogenic

tubular complexes is consistent with a role in islet neoformation, as is the increased number of insulin$^+$ ductal EIC observed following injections of exogenous CAMP/LL-37. In addition to intestinal expression, endogenous PLN- and islet-associated CAMP is also localized to sites that can influence T1D, by altering pancreatic immunity or endocrine pancreas homeostasis. Thus, besides its activity in the gastrointestinal tract, CAMP also influences the target tissue.

CAMP/LL-37 associated with islet formation and bacterial modification in BBdp rats: An exciting finding was that administration of exogenous CAMP/LL-37 promoted islet neoformation in BBdp rats, as reflected by an increased number of duct-associated insulin$^+$ EIC. We previously reported that injection of young BBdp rats with islet neogenesis-associated peptide (INGAP), a peptide derived from RegIIIδ, similarly increased insulin$^+$ ductal EIC (275). CAMP could represent a novel stimulator of islet neoformation. Endogenous CAMP detection in embryonic islets and neogenic tubular complexes is also indicative of a role in islet development. Furthermore, the presence of CAMP in islets from newly diagnosed patients with T1D suggests potential involvement in islet expansion following β-cell destruction. There is evidence of residual β-cells, possibly arising by a regenerative attempt, in subjects with recent onset T1D (257; 258) and even in patients with longstanding T1D (259). Thus, exogenous CAMP/LL-37 represents a potential therapeutic for stimulation of residual β-cell expansion in patients.

The role of AMPs in T1D is receiving increased research focus and new links have been uncovered revealing associations between dietary alteration of AMP status, small

intestinal inflammation, and T1D development. RegIIIγ is a C-type lectin produced in the small intestine that interacts with peptidoglycan, a critical cell wall component of Gram-positive bacteria (214; 332; 333). RegIIIγ has been implicated in limiting gut colonization by opportunistic pathogenic bacteria (334; 335). In a recent publication by Emani *et al*. in *Diabetologia*, *Reg3g* gene expression was decreased in the ileum of diet-protected HC-fed NOD mice (314). This is the only other report besides ours (103) describing dietary modification of a small intestinal AMP in an animal model of spontaneous T1D. Intriguingly, HC feeding in NOD mice also promoted an increased abundance of *Coriobacteriaceae* (314). In our BBdp rat studies, we also observed increased *Coriobacteria* in two distinct diabetes-protective settings: *Coriobacteriales* were more abundant in prospective fecal samples from diabetes-resistant BBdp rats; in addition, *Coriobacteriales* and *Coriobacteriaceae* were increased in BBdp rats injected with exogenous CAMP/LL-37. Taken together, these intriguing coincidences suggest that these bacteria could afford protection from T1D. The putative anti-diabetic effects of *Coriobacteria* could be analogous to the anti-inflammatory, diabetes-inhibiting effects of the probiotic strain *L. johnsonii* N6.2 previously reported in BB-DP rats (88). Intestinal CAMP, either directly via immune effect or indirectly via microbial modification, could directly shape the phenotype of immune infiltrates and by extension, exert an influence on activation of gut-originating β-cell-reactive lymphocytes.

Abnormal islet expansion in young BBdp rats associated with T1D promotion: Our unique PPx-based prospective study revealed that increased expression of islet-regenerative genes *Reg3a* and *Reg3b* was a feature of pre-diabetic BBdp pancreata. Increased *Reg3a/b*

expression in pre-diabetic rats is consistent with abnormally increased islet expansion in young diabetes-prone animals (122; 123). This abnormal growth-associated feature could represent a compensatory response to a low-grade pancreatic inflammation present in young pre-insulitic BBdp rats. Pro-inflammatory cytokines were previously reported to promote expression of pancreatic Reg proteins (281). Consistent with this possibility, increased $CD68^+$ macrophage-associated endothelial infiltration was observed in pre-diabetic BBdp pancreata; in addition, increased pro-inflammatory pancreatic gene expression was observed compared with prospective-resistant BBdp pancreata by immune-based PCR array screening. Abnormal islet growth in young diabetes-prone subjects could potentiate inflammation. In support of this view, there are reports in humans and rodents that REG proteins are T1D autoantigens (336-338). Thus, abnormal islet expansion in parallel with enhanced pancreatic inflammation were features of pre-diabetic (and pre-insulitic) BBdp pancreata, which could represent important early events leading to pronounced islet autoimmunity.

Integrative model for T1D pathogenesis: An overview of the major findings from the thesis and their proposed relation to T1D pathogenesis is presented in a simplified summary model (**Fig. 7.1**). Diabetes-prone rats display a deficit in anti-inflammatory $CD163^+$ M2 macrophages in the small intestine compared with control strains, which predisposes these animals to chronic gut inflammation (103). Exposure to cereal antigens promotes T cell infiltration in the small intestinal epithelium and lamina propria, resulting in establishment of an *Ifng*-associated T_H1 gene expression profile (103). Pro-inflammatory IFN-γ directly inhibits expansion of M2 macrophages, contributing to a further decrease in the number of $CD163^+$ cells and lower tolerogenic *Cd163* gene expression in cereal-fed animals (103). In parallel, the inflamed T_H1 phenotype impairs tight junction homeostasis, resulting in

enhanced gut leakiness and exacerbation of deleterious immune interactions with diabetogenic cereal antigens. Cereal feeding also impairs intestinal production of the antimicrobial peptide CAMP in association with the cereal-associated reduction in $CD163^+$ M2 macrophages, which represent important CAMP producers in the jejunum. $CD163^+CAMP^+$ cells are also present in PLN and this population could dampen pancreas-associated inflammation in addition to the jejunum. In the pre-diabetic pancreas, endothelium-associated $CD68^+$ macrophages become apparent shortly after weaning in association with enhanced pancreatic chemokine production. In parallel, abnormally enhanced islet expansion capacity, characterized by decreased β-cell cycle inhibition and enhanced expression of *Reg3a* and *Reg3b*, promotes T1D progression (123; 275). Overall, there is interaction among environment, small intestinal inflammation, and pancreatic homeostasis that converges to promote T1D in susceptible subjects. Cereal antigens promote T cell infiltration and T_H1 responses in the jejunum in parallel with local deficits in tolerogenic immune cells and these features reflect T1D development. Administration of exogenous CAMP/LL-37 can upregulate T1D-protective probiotics such as *Coriobacteria* and *Bifidobacteria*, which are associated with establishment of anti-inflammatory immune infiltrates in the jejunum. In the pancreas, CAMP/LL-37 can promote expansion of $insulin^+$ cells in diabetes-prone rats, which could compensate for β-cell loss following autoimmune destruction. CAMP/LL-37 represents a novel T1D candidate therapeutic with multifunctional effects associated with protection.

Conclusion

This research focused on the integrative biology of T1D pathogenesis in spontaneous rat models. The complexities of environmentally-modifiable T1D were explored with emphasis on the gastrointestinal immune system. This research provides a novel working model that incorporates key roles for gut lumen antigens, mucosal immunity, and the role of islets and altered regenerative capacity in T1D development. It is hoped this work will lead to prevention, treatment or even reversal of this devastating autoimmune disease.

FIGURE. 7.1

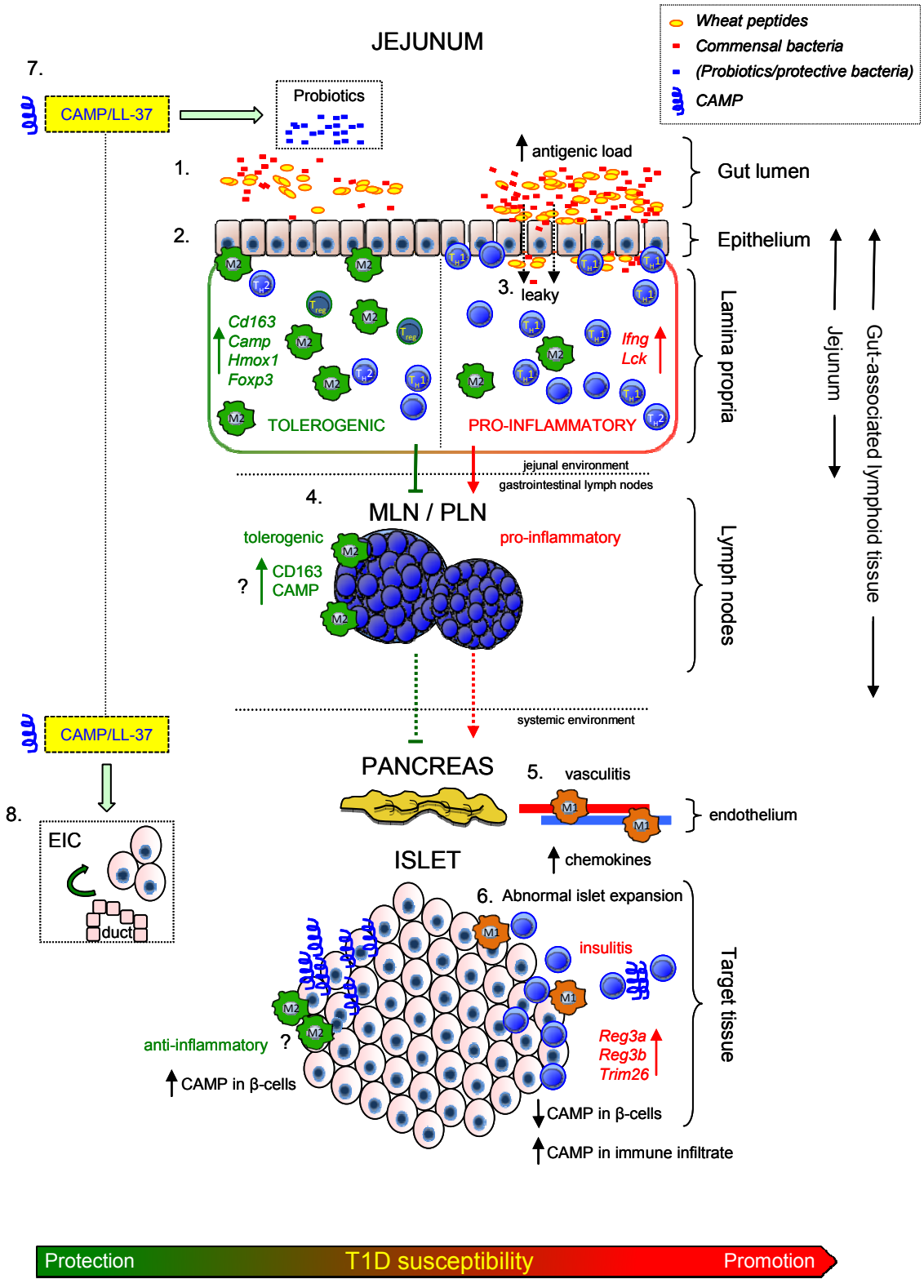

Wheat peptides
Commensal bacteria
(Probiotics/protective bacteria)
CAMP
JEJUNUM
7.
CAMP/LL-37
Probiotics
1.
antigenic load
Gut lumen
2.
Epithelium
3.
leaky
Cd163
Camp
Hmox1
Foxp3
TOLEROGENIC
Ifng
Lck
PRO-INFLAMMATORY
Lamina propria
Jejunum
Gut-associated lymphoid tissue
jejunal environment
gastrointestinal lymph nodes
4.
MLN / PLN
tolerogenic
pro-inflammatory
?
CD163
CAMP
Lymph nodes
systemic environment
CAMP/LL-37
PANCREAS
5.
vasculitis
endothelium
8.
EIC
duct
ISLET
chemokines
6.
Abnormal islet expansion
insulitis
anti-inflammatory
?
CAMP in β-cells
Reg3a
Reg3b
Trim26
CAMP in β-cells
CAMP in immune infiltrate
Target tissue
Protection
T1D susceptibility
Promotion

Figure 7.1. Integrative model of T1D pathogenesis
Major findings from the thesis are summarized in a simplified model of T1D development. (1) Environmental factors encountered in the jejunum, including cereal antigens (yellow ovals) and commensal microbes (red rectangles) influence spontaneous T1D development. (2) Diabetes-prone rats display an intestinal deficit in anti-inflammatory $CD163^+$ M2 macrophages compared with control strains, predisposing to aberrant inflammation in response to normally innocuous environmental determinants (103). Exposure to cereal antigens promotes enhanced T cell infiltration in the gut epithelium and lamina propria, leading to gut immune activation and establishment of a pro-inflammatory *Ifng*-associated T_H1 gene expression phenotype (103). Cereal-associated IFN-γ induction conversely inhibits M2 macrophage expansion, contributing to a further decrease in immune regulation, as reflected by decreased $CD163^+$ cells and lower tolerogenic *Cd163* gene expression (103). (3) In parallel, IEL infiltration and T_H1 activation impair gut barrier function, promoting gut leakiness and further increasing immune interaction with cereal antigens and exacerbating inflammation. Cereal feeding also impairs jejunal expression of the antimicrobial peptide CAMP. $CD163^+$ M2 macrophages represent important CAMP producers in the jejunum and this peptide could underlie some anti-inflammatory properties of these immunomodulatory cells. (4) $CD163^+CAMP^+$ cells are also present in PLN and could dampen pancreas-associated inflammation. (5) In the pre-diabetic pancreas, endothelium-associated $CD68^+$ macrophages become apparent shortly after weaning in association with enhanced pro-inflammatory chemokine production. (6) In parallel, abnormally increased islet expansion, featuring decreased β-cell cycle inhibition and enhanced expression of islet-regenerative genes *Reg3a* and *Reg3b*, promotes T1D progression (123; 275). Overall, cereal antigens promote T cell infiltration and T_H1 responses in the jejunum in parallel with local deficits in tolerogenic immune cells; these features reflect T1D development. (7) Administration of exogenous CAMP/LL-37 upregulated T1D-protective probiotics including *Coriobacteria* and *Bifidobacteria*. (8) In the pancreas, CAMP/LL-37 promoted expansion of duct-derived $insulin^+$ cells in diabetes-prone rats, which could provide a means of compensating for β-cell loss following autoimmune destruction. Thus, CAMP/LL-37 represents a novel T1D therapeutic candidate with multifunctional effects that could afford protection.

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STATEMENT OF CONTRIBUTION FROM COLLABORATORS

Dr. Gen-Sheng Wang, *Research Associate, Scott laboratory.* Performed staining of CAMP in human pancreata. Co-designed CAMP/LL-37 injection study and performed related neogenesis analysis (*Chapter 5*). Co-designed and performed CD3 staining in "Germ-Free" study (*Chapter 3*). Co-designed the PPx study and performed PPx surgeries (*Chapter 6*). Provided advice and assistance on immunohistochemistry experiments.

Dr. David E. Lefebvre, *Graduate student, Scott laboratory.* Participated in "Germ-Free" study (*Chapter 3*).

Jennifer A. Crookshank, *Senior Research Technician, Scott laboratory.* Participated in the "Germ-Free" and LEW-DP studies and generated related survival curves (*Chapters 3-4*). Provided technical advice for gene expression, cell culture, and immunoblotting experiments.

J. Ariana Noel, *Graduate student, Scott laboratory.* Generated gene list and performed bioinformatics for PPx microarray experiment (*Chapter 6*).

Dr. Alexander Strom, *Postdoctoral Fellow, Scott laboratory.* Provided assistance for insulin ELISA experiment (*Chapter 3*).

Dr. Majid Mojibian, *Graduate student, Scott laboratory.* Provided advice for T_{reg} analysis.

Dr. Lisa M. Kauri, *Postdoctoral Fellow, Scott laboratory.* Obtained collagenase-isolated islets (*Chapter 5*).

Chandra Eberhard, *Honours student, Scott laboratory.* Provided assistance and performed subset of gene expression experiments in "Germ-Free" study (*Chapter 3*).

Ariel Hendin, *Medical student, Scott laboratory.* Performed CD163 analysis in LEW-DP study (*Chapter 4*).

Turki Abujamel, Walid Mettawea (*Graduate students*)**, Alain Stintzi** (*Senior Scientist*). Processed fecal samples and performed microbiota analyses (*Chapter 5*).

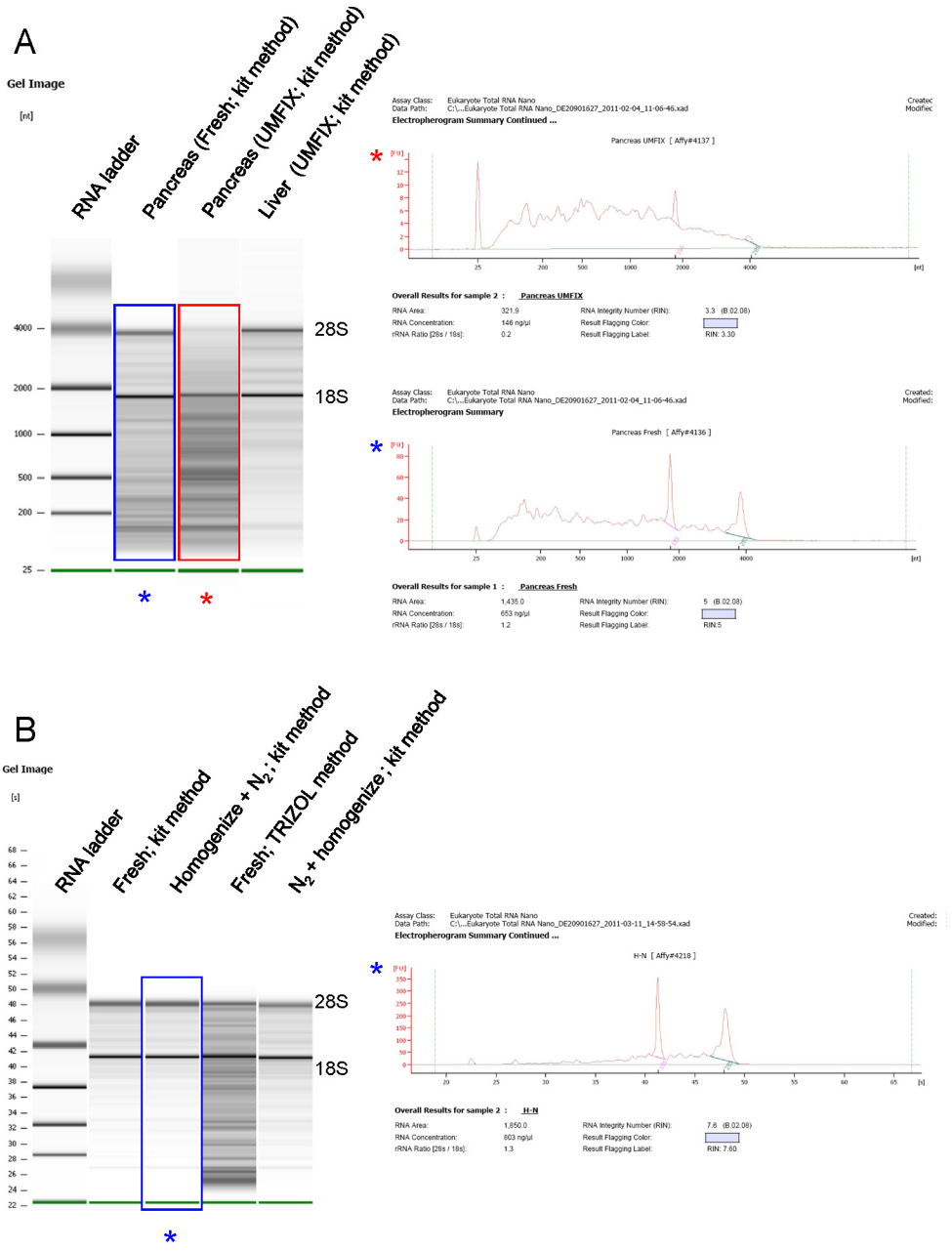
Caroline Vergette, *Senior Research Technician, OHRI Stemcore Laboratories.* Evaluated RNA for high-throughput gene expression experiments. Performed microarray experiments.

Garnet Rodger, Pierre Bradley, *Animal Technicians, OHRI Animal Care Unit.* Maintained animal colonies. Performed CAMP/LL-37 injections (*Chapter 5*). Assisted with PPx surgeries (*Chapter 6*).

Dr. Sigurd Lenzen, *Senior Scientist, Hannover Medical School.* Provided LEW-DP and LEW-C rats for foundation of Ottawa colony (*Chapter 4*).

Dr. Stephen J. Brooks, Dr. Martin L. Kalmokoff*, Senior Scientists, Health Canada.*
Performed animal work for "Germ-Free" study, including microbial monitoring (*Chapter 3*).

Appendix A.1.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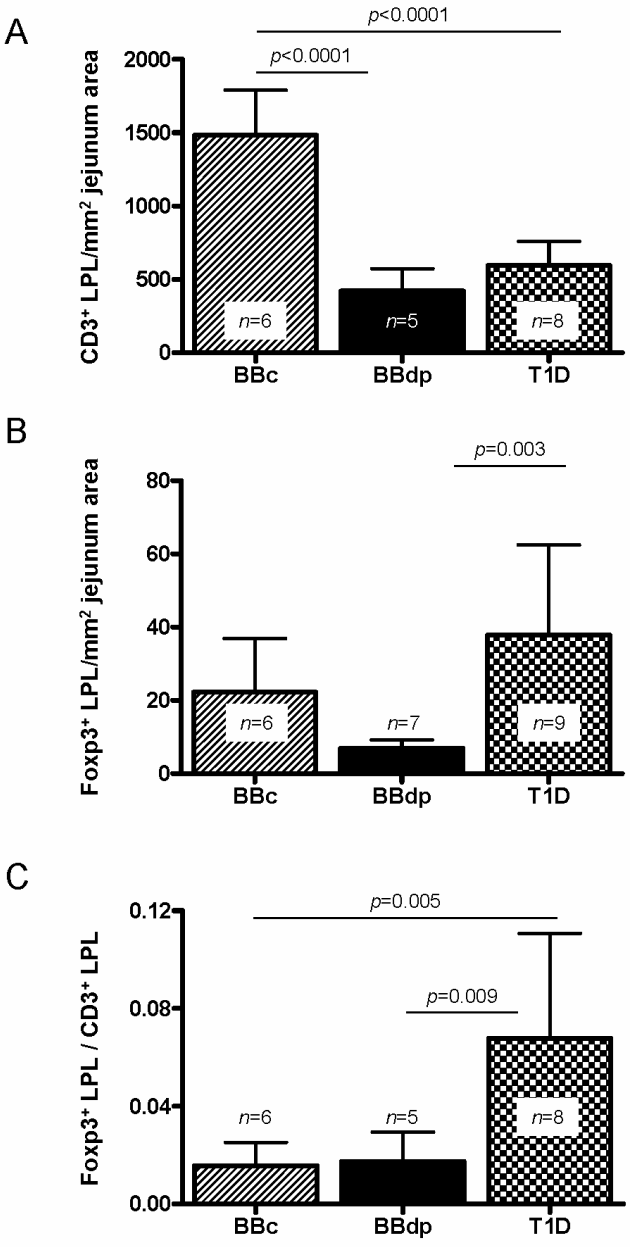
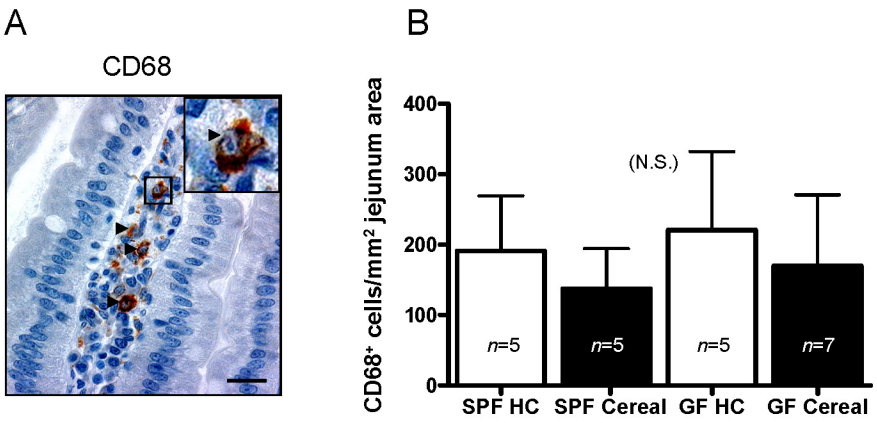
18S
Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE20901627_2011-02-04_11-06-46.xad
Electropherogram Summary Continued ...
Pancreas UMFIX [Affy#4137]
Overall Results for sample 2 : Pancreas UMFIX
RNA Area: 321.9
RNA Integrity Number (RIN): 3.3 (B.02.08)
RNA Concentration: 146 ng/µl
Result Flagging Color:
rRNA Ratio [28s / 18s]: 0.2
Result Flagging Label:
RIN: 3.30
Electropherogram Summary
Pancreas Fresh [Affy#4136]
Overall Results for sample 1 : Pancreas Fresh
RNA Area: 1,435.0
RNA Integrity Number (RIN): 5 (B.02.08)
RNA Concentration: 653 ng/µl
Result Flagging Color:
rRNA Ratio [28s / 18s]: 1.2
Result Flagging Label:
RIN:5
B
Gel Image
[s]
RNA ladder
Fresh; kit method
Homogenize + N2; kit method
Fresh; TRIZOL method
N2 + homogenize ; kit method
28S
18S
Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE20901627_2011-03-11_14-58-54.xad
Electropherogram Summary Continued ...
H-N [Affy#4218]
Overall Results for sample 2 : H-N
RNA Area: 1,850.0
RNA Integrity Number (RIN): 7.6 (B.02.08)
RNA Concentration: 803 ng/µl
Result Flagging Color:
rRNA Ratio [28s / 18s]: 1.3
Result Flagging Label:
RIN: 7.60

Appendix A.2.


A
p<0.0001
p<0.0001
CD3+ LPL/mm2 jejunum area
2000
1500
1000
500
0
n=6
n=5
n=8
BBc
BBdp
T1D
B
p=0.003
Foxp3+ LPL/mm2 jejunum area
80
60
40
20
0
n=6
n=7
n=9
BBc
BBdp
T1D
C
p=0.005
p=0.009
Foxp3+ LPL / CD3+ LPL
0.12
0.08
0.04
0.00
n=6
n=5
n=8
BBc
BBdp
T1D

Appendix A.3.


A
CD68
B
CD68⁺ cells/mm² jejunum area
400
300
200
100
0
(N.S.)
n=5
n=5
n=5
n=7
SPF HC
SPF Cereal
GF HC
GF Cereal

APPENDICES

Appendix A.1. Optimization of pancreatic RNA isolation for PPx study

Evaluation of RNA quality associated with different RNA isolation strategies. RNA quantity and integrity was evaluated on a 6000 Nano LabChip using an Agilent 2100 Bioanalyzer (OHRI Stemcore Laboratories). (**A**) Comparison of RNA quality from RNA extracted from fresh pancreas, pancreas fixed with UMFIX, and liver fixed with UMFIX; use of fresh pancreas resulted in decreased degradation compared with pancreas subjected to UMFIX-based fixation (**B**) Comparison of RNA extraction using different strategies; homogenization of pancreas tissue followed by flash freezing in liquid nitrogen and subsequent RNA isolation resulted in the least RNA degradation compared with other approaches.

Appendix A.2. Distribution of Foxp3$^+$ T_{reg} in BBc rats and BBdp rats

The effect of rat strain and disease status on CD3$^+$ cells and Foxp3$^+$ cells was evaluated in control BBc rats (hatched bars), asymptomatic BBdp rats (filled bars), and overtly diabetic BBdp rats (patterned bars). Effect of rat strain and disease status on (**A**) CD3$^+$ LPL and (**B**) Foxp3$^+$ cells in the small intestine of BBc and BBdp rats. (**C**) Effect of rat strain and disease status on the proportion of Foxp3$^+$ cells normalized to CD3$^+$ LPL in the jejunum. *n*=5-9 rats/group. Data represent mean ± SD. *P*-values obtained using ANOVA followed by LSD post-hoc test.

Appendix A.3. Effect of diet and microbes on CD68$^+$ cells in BBdp rats

CD68$^+$ cells were analyzed in the jejunum of 130 d asymptomatic BBdp rats housed in the presence or absence of microbes and fed diabetes-modifying diets; cereal diet (filled bars), HC diet (open bars). (**A**) Representative image displaying CD68 immunostaining in jejunum of cereal-fed BBdp rat; bar=20 μm. Arrows indicate labeled cells; inset shows digital magnification of boxed region. (**B**) Number of CD68$^+$ cells in jejunum of BBdp rats. *n*=5-7 rats/group. Data represent mean ± SD.

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Title or numeric reference of the portion(s)	Article title: "Promotion of autoimmune diabetes by cereal diet in the presence or absence of microbes associated with gut immune activation, regulatory imbalance, and altered cathelicidin antimicrobial Peptide"; requesting reuse of Figures 1-6
Title of the article or chapter the portion is from	Article title: "romotion of autoimmune diabetes by cereal diet in the presence or absence of microbes associated with gut immune activation, regulatory imbalance, and altered cathelicidin antimicrobial Peptide"
Editor of portion(s)	N/A
Author of portion(s)	N/A
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Thesis: *Cereal induced autoimmune diabetes is associated with small intestinal inflammation, downregulated anti-inflammatory innate immunity and impaired pancreatic homeostasis*

B.Sc. Honours, Biopharmaceutical Sciences, Genomics Option
Faculty of Science, University of Ottawa; graduated 2005 *cum laud*e
Thesis: *The effects of diet and islet neogenesis-associated protein (INGAP) on islet homeostasis in diabetes prone BioBreeding rats*

HONOURS & AWARDS

Queen Elizabeth II Graduate Scholarship in Science and Technology (*2*)
Ontario Ministry of Training, Colleges and Universities
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Ontario Graduate Scholarship in Science and Technology
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Ontario Graduate Scholarship (*3*)
Ontario Ministry of Training, Colleges and Universities
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University of Ottawa Excellence Scholarship
Faculty of Graduate and Postdoctoral Studies, University of Ottawa
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CIHR Travel Award
Nominated by Department of Biochemistry, Immunology and Microbiology (University of Ottawa) as representative for competition at 24th Annual Canadian Institutes of Health Research (CIHR)/Canadian Student Health Research Forum (CSHRF) National Poster Competition (2011, Winnipeg MB, Canada)

Graduate Studies Admissions Scholarship
Faculty of Graduate and Postdoctoral Studies, University of Ottawa
Value: Tuition. Funding period: 2006-2007

HONOURS & AWARDS (*continued*)

Dean's Scholarship for Excellence
Faculty of Science, University of Ottawa
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University of Ottawa Merit Scholarship
Value: $500. Award period: 2003-2004

Dean's Honour List
Faculty of Science, University of Ottawa
Award period: 2002-2004

PUBLICATIONS

Peer-reviewed articles:

Patrick C, Wang GS, Lefebvre DE, Crookshank JA, Sonier B, Eberhard C, Mojibian M, Kennedy CR, Brooks SP, Kalmokoff ML, Maglio M, Troncone R, Poussier P, Scott FW. Promotion of autoimmune diabetes by cereal diet in the presence or absence of microbes associated with gut immune activation, regulatory imbalance, and altered cathelicidin antimicrobial peptide. *Diabetes* 62(6): 2036-2047, 2013.

Sonier B, Strom A, Wang GS, **Patrick C**, Crookshank JA, Mojibian M, MacFarlane AJ, Scott FW. Autoantibodies from a patient with both type 1 diabetes and celiac disease bind to macrophages and monocytes expressing the scavenger receptor CD163. *Canadian Journal of Gastroenterology* 25: 327-329, 2011.

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Sonier B, **Patrick C**, Ajjikuttira P, Scott FW. Intestinal immune regulation as a potential diet-modifiable feature of gut inflammation and autoimmunity. Invited review for issue entitled: "Modulation of GALT function by diet components and drugs". *International Reviews of Immunology* 28(6): 414-45, 2009.

Presentations and abstracts at scientific meetings:

Patrick C*, Wang GS, Crookshank JA, Kennedy CR, Scott FW (2012). Cathelicidin Anti-Microbial Peptide (CAMP) - a potential islet trophic factor and biomarker of gut CD163 regulatory macrophages. **Poster presentation at the 12th International Congress of the Immunology of Diabetes Society, Victoria, BC, Canada*

Patrick C*, Crookshank JA, Wang GS, Noel A, Hendin A, Scott FW (2011). Decreased intestinal immune regulatory cells in autoimmune diabetes-prone LEW.1AR1-*iddm* rats - modification by diet. **Poster presentation at the 2nd International Conference on Immune Tolerance in Amsterdam, The Netherlands*

Patrick C*, Wang GS, Lefebvre DE, Crookshank JA, Mojibian M, Eberhard C, Scott FW (2011). Promotion of autoimmune diabetes and intestinal inflammation in cereal-fed diabetes-prone rats in the absence or presence of microbes. **Competed in poster presentation at the 24th Annual CIHR/CSHRF National Poster Competition, Winnipeg, MB, Canada*

Patrick C*, Wang GS, Crookshank JA, Eberhard C, Scott FW (2010). Decreased resident $CD163^+$ tolerogenic macrophages in $CD8^+$ T cell-infiltrated small intestine of cereal-fed diabetes-prone rats. **Platform presentation at the 11th International Congress of the Immunology of Diabetes Society, Incheon, South Korea; also presented orally at 10th Annual Ottawa Hospital Research Institute Research Day, Ottawa, ON, Canada*

Crookshank JA, **Patrick C**, Wang GS, Scott FW (2010). Dietary protection from type 1 diabetes development associated with increased $Foxp3^+$ cells in the gut immune system of the LEW.1AR1/Ztm-*iddm* rat. *Diabetes* 59 (Suppl. 1): A89, 2010. *American Diabetes Association 70th Scientific Sessions, Orlando, FL, USA*

Patrick C*, Wang GS, Lefebvre DE, Crookshank JA, Mojibian M, Scott FW (2008). Alterations in gut immune infiltrate parallel diet-related autoimmune diabetes in germ-free diabetes-prone rats. *Diabetes* 56 (Suppl. 1): A668, 2008. **Platform presentation at the American Diabetes Association 68th Scientific Sessions, San Francisco, CA, USA*

Patrick C*, Wang GS, Kauri LM, Crookshank JA, Rosenberg L, Scott FW (2007). Effects of INGAP on islet neoformation in diabetes-prone BioBreeding rats fed a protective hydrolyzed casein diet. The FASEB Journal. 2007; 21:866.15. **Poster presentation at Experimental Biology 2007, Washington, DC, USA*

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