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

Short Title: Cereal diet promotes T1D in the absence of microbes

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ABSTRACT

We are exposed to millions of microbial and dietary antigens via the gastrointestinal tract, which likely plays a key role in type 1 diabetes (T1D). We differentiated the effects of these two major environmental factors on gut immunity and T1D. Diabetes-prone BioBreeding (BBdp) rats were housed in specific pathogen-free (SPF) or germ-free (GF) conditions and weaned onto diabetes-promoting cereal diets or a protective low antigen hydrolyzed casein (HC) diet and T1D incidence was monitored. Fecal microbiota 16S rRNA genes, immune cell distribution, and gene expression in jejunum were analyzed. T1D was highest in cereal-SPF (65%) and cereal-GF rats (53%) but inhibited and delayed in HC-fed counterparts. Nearly all HC-GF rats remained diabetes-free whereas HC-fed SPF rats were less protected (7% vs. 29%). Bacterial communities differed in SPF rats fed cereal compared with HC. Cereal-SPF rats displayed increased gut CD3+ and CD8α+ lymphocytes, ratio of Ifng/Il4 mRNA, and Lck expression, indicating T cell activation. The ratio of CD3+ T cells expressing the Treg marker Foxp3+ was highest in HC-GF and lowest in cereal-SPF rats. Resident CD163+ M2 macrophages were increased in HC-protected rats. The cathelicidin antimicrobial peptide (Camp) gene was upregulated in the jejunum of HC diet-protected rats and CAMP+ cells co-localized with CD163. A cereal diet was a stronger promoter of T1D than gut microbes in association with impaired gut immune homeostasis.

Keywords: BB rat, CD163 macrophages, microbiota, cathelicidin, type 1 diabetes
INTRODUCTION

Despite extensive studies of the genetic and immune basis of human type 1 diabetes (T1D) over the past 50 years, neither cure nor prevention has been achieved (1). T1D occurs in individuals predisposed to β-cell-specific autoimmunity that is provoked by encounters with poorly understood environmental factors (2). Immune tolerance is impaired and islet regenerative capacity is limited, resulting in a deficit of β-cells. Thus, interventions that do not address the environmental component of this disease are likely to fail or have limited benefit as has been the case with clinical trials to date (3). A major gap in our understanding of T1D is the extent to which environmental factors encountered in the gastrointestinal tract influence disease outcome.

The gastrointestinal tract is the nexus of interaction between self and non-self. A large collection of immune cells defends the gut against pathogens while regulatory cells dampen inappropriate immune responses against dietary components and commensal bacteria. Accumulating evidence suggests the gut is structurally and immunologically abnormal in a subset of individuals prone to T1D (2; 4–6). For example, oral tolerance is frequently impaired in diabetes-prone rats (7) and humans (8). Gut inflammation (6) and leakiness are sometimes present and correcting this in diabetes-prone rats by closing epithelial tight junctions (5) or feeding a protective diet prevents T1D (2). When the gut is inflamed and leaky, unusually high concentrations of microbial and dietary antigens can activate immune cells in the gut associated lymphoid tissue from whence they traffic to the pancreatic lymph nodes (9).

Although we begin life germ-free, mammals are quickly colonized by a vast collection of microbes, the diversity of which is determined by ingested food (10). Cereal-based diets promote T1D in three rodent models of spontaneous T1D, NOD mice (11), diabetes-prone BioBreeding (BBdp) rats (reviewed in ref (2)), and diabetes-prone LEW.1AR1/Ztm-iddm rats (12). In all three models, feeding a low antigen, hydrolyzed casein (HC)–based diet protects against T1D (2) and this may also be the case in humans (13). A newly diagnosed child fed a gluten-free diet has remained without insulin therapy for 20 months (14). Establishing oral tolerance early to a cereal diet or wheat proteins alone protects BBdp rats from T1D (15). Thus, food has a major effect on expression of T1D (2) and recent evidence also implicates the gut microbiota (16). The gut lumen contains large amounts of food molecules that affect gut immunity (7; 8), islet mass (17) and strongly influence the composition of the microbiota. Most studies have focused on either diet or microbes, but not both. Gastrointestinal illnesses were linked to islet autoimmunity only in those exposed to wheat and barley in early childhood (18). Therefore, it is important to differentiate the contribution of dietary antigens and microbes to gut immune activation and T1D.

A major obstacle in understanding the importance of diet and gut inflammation to T1D is the overlap between intestinal immune response elicited by dietary antigens and microbes, which confounds interpretation of diet-specific modulation of T1D. In the present study, we established a colony of GF BBdp rats and compared them with BBdp rats housed under standard SPF conditions. We asked whether the effects of diabetes-modifying diets on T1D incidence could be observed independently of microbes and whether certain aspects of the gut immune system are modulated by diet and/or microbial exposure.
MATERIALS AND METHODS

Animals  Animal studies were approved by the local animal care committees. BBdp or control BBc rats were maintained in SPF or GF conditions and weaned onto the AIN93G hydrolyzed casein-based (HC) diet or a cereal diet (NTP-2000 fed to SPF rats or irradiated C.R. Rodent 18% diet (PMI Nutrition International, Brentwood, MO) fed to GF rats) beginning at 23 d. GF status was confirmed by microbiological and immunological testing, as well as PCR analysis. Animals were monitored biweekly for T1D defined as ≥ 11 mmol/L fasting blood glucose. Within 24-48 hours of T1D diagnosis or after ~134 d without T1D development, rats were euthanized. Pancreas and washed gut tissues were either fixed in Bouin’s fixative for histological evaluation or frozen for RNA isolation and gene expression analysis. Additional studies were performed using rats that were non-lymphopenic or which had in addition the Gimap5 (lymphopenia) gene inserted (19). Except where noted, all analyses were performed on pancreas or jejunum of BBdp rats that remained asymptomatic until ≥134 d.

Human subjects  Biopsies of jejunum were obtained from non-diabetic control subjects (patients with short stature, failure to thrive, gastroesophageal reflux disease, recurrent abdominal pain, anemia, n=14 males) and 8 male patients with T1D between 8-16 years of age with informed consent (20) All biopsy samples (1-7 per subject) on a single slide were analyzed (17).

Immunohistochemistry and morphometric analyses  Antibodies to the following markers were used: CD3 (Abcam), CD8α (BD Biosciences), CD4 (Abcam), Foxp3 (eBioscience), CD163 (rat; Santa Cruz Biotechnology), CD163 (human; Abcam), CAMP (Abcam), CD68 (AbD Serotec), and CD14 (Santa Cruz Biotechnology). Coded sections were analyzed using a Zeiss Axioplan2 microscope and Northern Eclipse morphometry software (Empix Imaging) (17). The numbers of CD3+, CD8α+, and CD4+ IEL were expressed as either the number of intra-epithelial lymphocytes (IEL) per 100 epithelial cells or lamina propria lymphocytes (LPL) per mm² mucosal area. Foxp3+ cells, CD163+ cells, and CD68+ cells were expressed as the cell number per mm² mucosal area. Corresponding biotinylated secondary antibodies (DAKO Diagnostics) were applied, followed by incubation with strepavidin-conjugated horseradish peroxidase (DAKO Diagnostics) and 0.06% dianaminobenzidine and 0.03% H2O2 as substrates; counterstain was hematoxylin. Immunofluorescence was performed using a Zeiss LSM 510 Meta confocal microscope and Alexa-488, Cy-3 and Cy-5-conjugated secondary antibodies (Life Technologies). Hoechst (Sigma) was used for nuclear staining.

Gene expression by real-time PCR  RNA was extracted from frozen jejunum using TRIzol (Invitrogen) or Nucleospin RNA II RNA Isolation Kit (Macherey-Nagel). Quantitative PCR studies were performed using TaqMan Gene Expression Assays (Life Technologies). An ABI Prism 7000 Sequence Detection System was used to obtain threshold cycle (Ct) values; results are shown as fold-change (2^-ΔΔCt). Changes in gene expression are presented in relation to the low antigen, HClGF group, which was arbitrarily set to one. For PCR array profiling, gene expression was analyzed using Innate and Adaptive Immune Response PCR arrays (SABiosciences/Qiagen) on samples from HC-SPF and cereal-SPF BBdp rats (n=4/group). Ct values were normalized with the average Ct values of five housekeeping genes.
**Statistics** Survival analysis was performed using the log rank test. Comparisons among sample populations were made using Student’s *t*-test or one-way ANOVA and least significant difference (LSD) post-hoc testing to determine the significance of differences between multiple means. For human samples, Mann-Whitney *U*-test was performed. *P*-values < 0.05 were considered statistically significant.
RESULTS

Feeding a cereal diet promotes T1D in the presence or absence of microbes As we previously reported in SPF BBdp rats (reviewed in (2)), feeding an HC diet increased age at onset and inhibited T1D development compared with a cereal diet (29% vs 65%, p<0.005) (FIG. 1A). The HC diet was even more protective in GF BBdp rats where T1D was almost completely prevented (7% vs 53% in cereal-fed GF rats, p<0.005). The main effect of microbes on T1D incidence was observed in HC-fed rats, in which disease was promoted by microbial exposure (HC-SPF, 29% vs. HC-GF, 7%, p<0.05). Cereal-fed rats housed in GF conditions displayed a delay in age at onset but T1D incidence was not significantly different compared with cereal-SPF rats. Thus, in the absence of an antigenic diet (HC feeding), microbes can promote T1D in BBdp rats whereas microbial exposure did not significantly enhance T1D progression in cereal-fed animals. Overall, our results demonstrate that microbes were not required for T1D development and cereal antigens were the strongest environmental promoters of T1D in the BBdp model.

Body mass, pancreatic mass and islet inflammation (insulitis) were not modified by microbes or diet in 130 d asymptomatic rats (data not shown). Total islet number was increased in GF rats compared with SPF rats (FIG. 1B), consistent with the delay in T1D onset in GF rats, regardless of diet. Under SPF conditions, rats fed an HC diet had a significantly increased β-cell mass compared with cereal-fed animals, consistent with our previous report (17). Cereal-fed rats raised in GF conditions displayed a larger β-cell mass compared with SPF cereal-fed rats. Among HC-fed rats, β-cell mass was not different between those raised in GF compared with SPF conditions. β-cell mass was lowest in cereal-SPF rats, and this was overcome by feeding the protective HC diet (HC-SPF). Serum insulin concentration was not different among the groups (data not shown).

Denaturing gradient gel electrophoresis of fecal samples from BBc and BBdp rats was performed to ascertain whether the bacterial communities were affected by diet. Non-metric multidimensional scaling analysis revealed that BBdp rats fed a protective HC diet had a distinct bacterial profile compared with either BBdp rats or control BB (BBc) rats fed a standard cereal diet (FIG. 1C).

Influence of diet and microbes on gut T cells Because gut inflammation has been reported in BBdp rats, NOD mice and human T1D patients, we evaluated the effect of diet and/or microbes on key gut immune cell populations. The highest numbers of both CD3+ and CD8α+ IEL and LPL occurred in cereal-SPF rats. HC-SPF rats displayed decreased CD3+ and CD8α+ cell numbers compared with cereal-SPF rats (FIG. 2A,B). No significant diet-related differences in the numbers of CD3+ or CD8α+ IEL or LPL were observed in GF animals. Among cereal-fed rats, the numbers of CD3+ and CD8α+ IEL and LPL were larger in SPF rats compared with GF rats. Also, there were more CD3+ cells in HC-SPF compared with HC-GF animals. Thus, CD3+ and CD8α+ lymphocytic infiltration was promoted by microbial exposure. The number of CD4+ cells was higher than expected and the overall pattern differed from CD3 and CD8α labeling, possibly due to other cell populations being labeled (FIG. 2C).

Foxp3 is a master transcription factor which directs the differentiation and function of regulatory T lymphocytes (Foxp3+ Treg)(21). Foxp3+ cells were exclusively localized to the
lamina propria (FIG. 3A) and confocal microscopy confirmed nuclear expression in CD3+ lymphocytes (data not shown). Unlike total CD3+ cells, the number of Foxp3+ cells did not decline under GF conditions. The most protective low antigen combination of GF housing and HC feeding resulted in the highest number of Foxp3+ cells (FIG. 3B) and this difference became significant when expressed as a percentage of jejunal CD3+ (FIG. 3C) or CD4+ LPL (FIG. 3D). A much lower percentage of Foxp3+ cells was observed in the most diabetes-promoting, high antigen situation of SPF housing and/or cereal feeding (FIG. 3C,D). Thus, the dietary influence on Foxp3+ Treg proportion was apparent under sterile conditions, with protective HC feeding resulting in enrichment of regulatory lymphocytes.

The pro-inflammatory Th1 cytokine gene, Ifng was upregulated by cereal diet in GF rats (FIG. 4A). Microbes increased Ifng expression in HC-SPF rats vs. HC-GF rats but expression was similar in cereal-SPF and cereal-GF rats. There were no differences in Il17a gene expression among the four groups (FIG. 4B) and II15 expression was largest in cereal-GF rats (FIG. 4C). Under GF conditions, the cereal diet stimulated upregulation of the hallmark Th2 cytokine, Il4 compared with HC-feeding (FIG. 4D). The ratio of Ifng/Il4 was highest in cereal-SPF rats and this was significantly different from the HC-SPF and cereal-GF groups (FIG. 4G), reflecting enhanced cereal-associated Th1 polarization in the gut immune system as reported previously (7). The highest expression of Il10 and Tgfb1 was in SPF compared with GF rats, regardless of diet (FIG. 4E,F). The ratio of Ifng/I10 was highest in cereal-GF rats and low in the other three groups (FIG. 4H). The ratio of Ifng/Tgfb1 was highest in cereal-GF rats and was relatively low in the other three groups (FIG. 4I). The data suggest that overall, antigenic stimulation by microbial exposure and/or cereal feeding upregulated cytokine gene expression, with Ifng expression predominating over various counter-regulatory cytokines in cereal-fed rats.

**Increased frequency of CD163+ macrophages in diet-protected rats**  
Tissue-resident CD163+ (M2) macrophages are immunosuppressive, have lower levels of MHC class II and other markers of classically-activated macrophages (22) and contribute to maintenance of a hyporesponsive state. We observed CD163+ cells mainly in the lamina propria and also in a smaller subset in the epithelial compartment (FIG. 5A). In a previous analysis of cereal-fed SPF BBdp rats, we observed a deficiency in gut CD163+ macrophages compared with control BBc rats (FIG. 5B). In the present study of BBdp rats only, feeding the protective HC diet increased the number of CD163+ cells compared with BBdp rats fed a cereal-based diet. (FIG. 5C). No dietary differences were observed under GF conditions. Gene expression analysis was consistent with these data (FIG. 5D). The Hmox1 gene for the rate-limiting cytoprotective heme oxygenase-1 (HO-1) enzyme in the CD163 pathway, showed highest expression in the most protected animals lacking antigen exposure, HC-GF (FIG. 5E). The number of CD68+ macrophages was unaffected by either diet or microbial status (data not shown). These data suggest the influence of the environment on macrophages occurs primarily through changes in the M2 subset of macrophages, in keeping with the HC-associated decrease in Ifng/Il4 ratio (FIG. 4G), which favours polarization of M2 macrophages (23). To investigate the potential relevance of these results to human patients, we performed preliminary analysis of gut biopsies from a small group of male children newly diagnosed with T1D. This analysis revealed lower numbers of CD163+ macrophages in the jejunum compared with control individuals (FIG. 5F,G).
Upregulation of *Camp* in diet-protected BBdp gut associated with CD163⁺ M2 macrophages

To gain a better understanding of dietary modulation of immune factors under standard housing conditions, we screened for diet-modifiable immune factors in the jejunum of conventionally-housed SPF rats using focused immune-associated PCR arrays. Only candidates that were differentially expressed ±2 fold with *p* <0.05 were explored; results are presented as a volcano plot (FIG. 6A). *Il1f6* expression was 2.78-fold upregulated in HC-fed rats compared with cereal-fed rats (*p*=0.004) (FIG. 6B). *Il1f6* encodes the cytokine interleukin 1 family member 6. The IL-1F6 cytokine has been implicated in cutaneous inflammation (24) and was found to induce antimicrobial expression, including beta-defensins and cathelicidin antimicrobial peptide (CAMP) (25). Interestingly, the other significantly upregulated gene in HC-fed BBdp rats was *Camp*. *Camp* expression was 2.37-fold upregulated in HC-fed rats compared with cereal-fed rats (*p*=0.03) (FIG. 6B). CAMP is a multifunctional antimicrobial effector and immunomodulatory host defense factor (26). In addition, consistent with increased CD3⁺ and CD8α⁺ cells in gut of cereal-fed rats (FIG. 2), the expression of Lck, a T cell signaling molecule, was 2.43-fold down-regulated in HC-fed rats compared with cereal-fed rats (*p*=0.003) (FIG. 6B). CAMP⁺ cells were detected in the epithelium and lamina propria of the SPF jejunum (FIG. 6C). CAMP⁺ cells were also present in gut of sterile embryos and GF BBdp rats (FIG. 6C), suggesting CAMP is not only antimicrobial but has additional functions. Additional confocal analyses revealed that CAMP co-localized with CD163⁺ and CD14⁺ cells in the epithelium and lamina propria (FIG. 6D), but not with the macrophage marker, CD68 (FIG. 6C). This suggests that CAMP could be an effector peptide or product of M2 macrophages. These findings reveal that feeding a protective HC diet is associated with antimicrobial gene upregulation in CD163⁺ macrophages in the small intestine.

Gut inflammation in BBdp rats is not due to lymphopenia or diabetes risk MHC

We previously reported that BBdp rats display enteropathy that precedes T1D and is not present in control BBc rats (6). To determine whether BBdp enteropathy is linked to major BBdp diabetes risk loci, we evaluated the presence of enteropathy in various congenic rat strains. Jejunal sections of congenic ACI.1⁺ and ACI.1⁺lyp/lyp rats were analyzed at 49, 60, 82 and 259 d. There were no distinct morphological abnormalities observed in the gut tissue from these congenic animals (FIG. 7). Inflammation was absent in non-lymphopenic rats congenic for the diabetes risk MHC II allele, RT1⁺ (Wistar Furth, BB control, ACI.1⁺) nor was it present in ACI.1⁺.lyp/lyp rats that possess both the RT1⁺ allele and the Gimap5 mutation. Therefore, the presence of enteropathy in BBdp rats was not attributable to Gimap5-associated lymphopenia or the rat diabetes risk MHC, RT1⁺.
DISCUSSION

The relationship among diet, microbiota, gut immunity and diabetes is poorly understood and at times contradictory. Some studies report no relation between early infections or vaccinations and risk of T1D (27) and a large nationwide study of Danish children found no association with antibiotic use (28). MyD88-deficient NOD mice were protected from T1D under SPF conditions but not GF conditions, further illustrating the complex role of microbes in T1D (16).

In the present study, a cereal-based diet was the major diabetes-promoting factor in both SPF and GF conditions. This clearly illustrates that despite dietary differences in gut bacterial profiles (FIG. 1C), diet has a stronger influence than microbes on T1D development in these animals. An earlier report also found that microbes were not essential for development of T1D in the cereal-fed BB rat (29). The near complete protection afforded by the HC diet in GF conditions was partially inhibited in the presence of microbes (SPF-HC). Thus, microbes promote T1D, but to a lesser extent than cereal diets. Among HC-fed rats, microbes also promoted infiltration of T cells (FIG. 2) and decreased the proportion of Tregs (FIG. 3). It is possible that any effect of bacteria would be most prominent in the colon (30). Immune system changes in the jejunum may only be part of the picture and a more detailed examination of the influence of microbial and dietary antigenic load along the entire length of the gastrointestinal tract would also be informative.

Snell-Bergeon et al., suggest that diet-induced gut inflammation could be a prerequisite for gut pathogen-induced islet autoimmunity (18). Interestingly, we observed more overlap between the bacterial profiles of cereal-fed BBdp rats and cereal-fed control (BBc) rats compared with HC-fed BBdp rats. This suggests that HC feeding did not afford protection from T1D in SPF rats by shifting the bacterial profile toward the control strain profile. Nonetheless, the microbiota are different in BBDP rats that develop diabetes (31-33) and administering protective bacteria can prevent T1D (34). When BBDP Wor/Gro rats were treated with antibiotics and fed an HC diet from weaning, T1D was completely prevented (31), in agreement with the present results (FIG. 1) and consistent with the proposition that (gut-derived) environmental antigenic load is a key determinant of T1D promotion. The HC diet can change the gut microbiota (33), and this may contribute to the diabetes-protective effect. Thus, diet and microbes could have synergistic effects on gut immunity, islet homeostasis and T1D incidence.

Under SPF conditions, there were increases in the numbers of CD3+ and CD8α+ LPL and IEL in rats fed a cereal diet compared with an HC diet, demonstrating that under normal conditions, cereal feeding drives T cell infiltration in the small intestine of BBdp rats. While the cytotoxic nature of IEL in BBdp rats is unclear, it is known that patients with celiac disease, Crohn’s disease, and ulcerative colitis have increased densities of these cells that may mediate tissue damage in the small bowel (35), potentially increasing gut leakiness. When small intestinal biopsy samples from children with T1D were cultured with gliadin, the numbers of CD3+ IEL and activated lamina propria CD25+ cells were increased in T1D biopsies (20). These data indicate that inflammation is present in the jejunum of patients with T1D and suggest that the small intestine of subjects with T1D is immunologically more responsive to cereal antigens, consistent with our findings.
Ifng was upregulated by both microbes and cereal diet whereas Il10 and Tgfb were upregulated primarily by microbes. In GF rats, the cereal diet upregulated Il15, Il4, Ifng/Il10, and Ifng/Tgfb1. IL-15 is a pro-inflammatory cytokine that is implicated in celiac disease (35) and has recently been shown to promote T1D in NOD mice (36). In the HC-GF group, which had essentially no exposure to environmental antigens, gene expression for Ifng, Il4, and Il10 was low, corresponding with the decreased number of T cells in this group (FIG. 2). A combined effect of microbes and cereal feeding on pro-inflammatory Th1 polarization was evident from the ratio of Ifng/Il4, which was highest in the cereal-SPF group but low in the cereal-GF group. This ratio was decreased by HC feeding, further demonstrating an inhibition of the usual Th1 bias in the gut of BBdp rats (37). Thus, cereal antigens promote Th1-biased gene expression in the small intestine, as suggested previously (37). The induction of small intestinal T cell signaling in the cereal-SPF group was further reflected by increased expression of Lck, the most strongly expressed cereal-induced gene on the PCR array. Lck encodes a lymphocyte-specific protein tyrosine kinase/p56 involved in the initiation of T cell activation (38).

The involvement of Th17 in BB diabetes is controversial (39; 40). However, our data demonstrate no diet or microbe-induced differences in jejunum Il17a gene expression (FIG. 4B).

Foxp3+ T_reg play a central role in the inhibition of autoimmunity and suppression of physiological immune responses (41). In GF conditions, the proportion of Foxp3+ T_reg cells was decreased in cereal-fed rats compared with HC-fed rats. Strikingly, the proportion of Foxp3+ T_reg cells was lowest in the cereal-SPF rats and highest in the HC-GF rats reflecting the highest and lowest external antigen load and the highest and lowest T1D incidences, respectively. In addition, HC-GF rats displayed a significantly increased proportion of Foxp3+ T_reg compared with HC-SPF rats, which paralleled the delay in age of onset in GF rats. Consistent with this, Peyer’s patches of BALB/c mice fed a gluten-containing diet displayed a lower proportion of Foxp3+ T cells compared with mice fed a gluten-free formula (42). The decreased proportion of Foxp3+ T_reg in cereal-GF rats compared with HC-GF rats could be an important permissive factor contributing to pro-inflammatory reactivity to cereal antigens under sterile conditions. The relative increase in Foxp3+ T cells in HC-GF rats was not accompanied by increased gene expression for Tgfb1 and Il10. However, the lowest number of lamina propria CD4+ T cells was observed in HC-GF rats (FIG. 2C), by a factor of two to three fold, yet the levels of Tgfb1 in these animals were not different from cereal-GF rats and only half that of both SPF groups. We speculate that on a cell per cell basis the synthesis of Tgfb1 was increased in the HC-GF rats, further consistent with the high proportion of Foxp3+ T cells being the source of Tgfb1 (FIG. 3).

The largest reservoir of tissue resident regulatory macrophages in the body occurs in the lamina propria of the intestine (43). These cells suppress inflammation (44) and express CD163, a scavenger receptor for haptoglobin-hemoglobin complexes. CD163 is inducible by IL-4 and IL-10 (45) and differentiation of regulatory CD163+ macrophages is promoted by Foxp3+ T_reg (46). Thus, development and maintenance of an immunoregulatory state in the gut depends on a constant interplay between tolerogenic subsets of antigen presenting cells and T cells. The number of resident CD163+ M2 macrophages was inversely related to T1D risk, highest in control, diabetes-resistant BBc and lowest in overt diabetic BBdp rats and was increased in the diet-protected HC-SPF BBdp rats. The HC diet-induced increase in CD163+ cells required the presence of microbes. CD163 expression was highest when the ratio of Ifng/Il4 was low, consistent with a downregulation of Th1 inflammation by M2 macrophages. In addition, the
Hmox1 gene was down-regulated in antigen exposed groups and was highest in the most protected (low antigen) HC-GF group, suggesting additional environmental sensitivity of the CD163/HO-1 pathway (47). These findings are in keeping with a recent report that IL-4/IL-10/TGF-β–induced M2 macrophages prevented T1D in NOD mice (48). Furthermore, our preliminary data suggest there can be fewer CD163+ cells in the gut of some newly diagnosed T1D patients, a finding that requires confirmation.

The increase in small intestinal Camp gene expression in HC diet-protected BBdp rats and co-localization of CAMP and CD163 proteins suggests that antimicrobial peptide production is a function of M2 macrophages that could promote an anti-inflammatory state in the gut. Rats and humans possess only one cathelicidin gene (49). CAMP has been reported to be produced by neutrophils, monocytes, macrophages, and epithelial cells in various tissues (50). The most extensively characterized function of CAMP is microbicidal activity but additional roles have been described, including regulation of inflammation, growth, chemotaxis, tissue repair, wound healing, apoptosis regulation, and angiogenesis (49). CAMP has been implicated in inflammatory bowel diseases, as decreased CAMP expression has been reported in patients with Crohn’s disease (51). CAMP-deficient mice displayed a more pronounced form of colitis compared with control mice treated with dextran sodium sulfate (DSS) (52). In addition, intracolonic treatment with synthetic CAMP prevented DSS colitis, an effect associated with a decreased number of fecal bacteria, enhanced mucin production, and suppression of neutrophil infiltration (53). Thus, decreased Camp expression, partly from CD163+ M2 macrophages, could favor chronic inflammation induced by environmental antigens.

Therefore, in BBdp rats, a cereal diet promoted T1D in the presence or absence of microbes. Cereal feeding was associated with increased T cell infiltration, a TH1 cytokine bias, and deficiencies in anti-inflammatory CD163+ M2 macrophages which were partly corrected in SPF animals fed a protective HC diet. PCR array screening of SPF BBdp rat jejunum further revealed Camp as a novel HC diet-upregulated factor. CAMP co-localization with CD163 suggests it may be a new marker of M2 macrophages, and highlights a potential role for this antimicrobial peptide in T1D modulation. Importantly, the upregulation of CD163 in HC-SPF animals suggests that this protective effect required the presence of microbes, further emphasizing the complex interaction between diet and microbes in the gut of diabetes-prone animals. We speculate that diet-induced T1D depends on an increased load of antigens from diet and/or microbes crossing a leaky gut epithelial barrier and activating β-cell-specific immunity. This immune activation was dampened when animals were fed an essentially antigen-free HC diet, particularly under germ-free conditions. Microbes could modify this process as the HC diet resulted in a different bacterial profile in parallel with increased CD163 macrophages that produce antimicrobial CAMP. This study highlights the fact that development of T1D is a heterogeneous, environmentally-driven process, particularly in humans, and is likely to occur by several different pathways.
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Fraser W. Scott conceived and designed the study and analyzed the data. Christopher Patrick, Gen-Sheng Wang, David E. Lefebvre, Jennifer A. Crookshank, Brigitte Sonier, Chandra Eberhard, Majid Mojibian, Stephen P.J. Brooks and Martin L. Kalmokoff designed and performed the experiments and analyzed the data. Mariantonia Maglio and Riccardo Troncone contributed human samples and helped write the paper. Fraser W. Scott, Christopher Patrick, Gen-Sheng Wang, David E. Lefebvre and Jennifer A. Crookshank wrote the paper. Christopher Kennedy contributed materials and analysis tools. Philippe Poussier contributed congenic animals, assisted in design of the study and helped write the paper.
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FIGURE LEGENDS

FIG. 1 Feeding a cereal diet promotes T1D in BBdp rats in the presence or absence of microbes – diabetes incidence, islets and bacterial communities BBdp rats were maintained under SPF or GF conditions, fed an HC diet or cereal diet, and monitored for T1D development. (A) Kaplan-Meier plot of percent non-diabetic rats. HC-GF group (n=27), HC-SPF group (n=48), Cereal-GF group (n=17), and Cereal-SPF group (n=48). (B) Islet number and β-cell mass in asymptomatic 130 d BBdp rats fed either an HC diet (open bars) or cereal diet (hatched bars) and housed under GF or SPF conditions. Data represent mean ± SD; n=5-7/group. P-values obtained using ANOVA followed by LSD post-hoc test. (C) Non-metric multidimensional scaling diagram of microbial community distribution in BBc rats fed cereal (open triangles), BBdp rats fed cereal (filled circles) or HC (open circles). The figure shows the relative statistical differences between bacterial communities derived from each sample on an arbitrary 2 dimensional surface. Similar community profiles are closer together in two dimensional space.

FIG. 2 Influence of diet and microbes on gut T cells BBdp rats were maintained under SPF or GF conditions and fed either an HC diet (open bars) or cereal diet (hatched bars). (A) Representative image displaying CD3+ intra-epithelial lymphocyte (IEL) and lamina propria lymphocytes (LPL) in BBdp jejunum, bar = 20 µm; number of CD3+ LPL/mm² mucosa and CD3+ IEL per 100 epithelial cells in 130 d asymptomatic BBdp rat jejunum. (B) Representative image displaying CD8α+ cells in BBdp jejunum, bar = 20 µm; number of CD8α+ LPL/mm² mucosa and CD8α+ IEL per 100 epithelial cells. (C) Representative image displaying CD4+ cells, bar = 20 µm; number of CD4+ LPL/mm² mucosa and CD4+ IEL per 100 epithelial cells. Data represent mean ± SD; n=5-7/group. P-values obtained using ANOVA followed by LSD post-hoc test.

FIG. 3 Regulatory Foxp3+ T lymphocytes (A) Representative image displaying Foxp3+ cells in BBdp jejunum, bar = 20 µm. (B) Number of total Foxp3+ cells/mm² mucosa, (C) number of Foxp3+ cells/mm² mucosa normalized to the number of CD3+ and (D) normalized to CD4+ LPL/mm² mucosa in BBdp rat jejunum. Data represent mean ± SD; n=7-9 rats/group. P-values obtained using ANOVA followed by LSD post-hoc test.

FIG. 4 Cytokine gene expression Frozen jejunal samples were obtained from BBdp rats maintained under SPF or GF conditions and fed either an HC diet (open bars) or cereal diet (hatched bars). RNA was isolated and gene expression analyses were performed by using reverse transcription followed by quantitative RT-qPCR. The ∆Cₜ value was obtained by subtracting the Ct value of β-actin from the gene of interest and the triplicate ∆Cₜ values were averaged for each animal. The ∆Cₜ value of the standard WF rat was subtracted from each animal to obtain the ∆∆Cₜ value. Results are shown as relative amounts 2^(-∆∆Cₜ) using an age-matched cereal-fed Wistar Furth (WF) rat as a standard; genes of interest were normalized to expression of β-actin. The average value obtained from the HC-GF group was standardized to 1 and averages from other groups were standardized by the same factor. Gene expression of (A) Ifng, (B) Il17a, (C) Il15, (D) Il4, (E) Il10, and (F) Tgfb1. Cytokine expression ratios are presented in (G) Ifng/Il4, (H) Ifng/Il10 and (I) Ifng/Tgfb1. Data represent mean ± SEM (boxes) ± SD (whiskers); n=5-7/group. P-values obtained using ANOVA followed by LSD post-hoc test.
FIG. 5  CD163⁺ M2 macrophages in BBdp jejunum  BBdp rats were maintained under SPF or GF conditions and fed either an HC diet (open bars) or cereal diet (hatched bars). (A) Representative image displaying CD163⁺ cells in BBdp jejunum, bar = 20 µm. (B) Number of CD163⁺ cells/mm² mucosa in jejunum of 130 d control BBc, 130 d asymptomatic BBdp and ~100-130 d overt diabetic BBdp rats, n=6-8. (C) Number of CD163⁺ cells/mm² mucosa in 130 d asymptomatic BBdp rat jejunum. Data represent mean ± SD; n = 7-9 rats/group. Gene expression of (D) Cd163 and (E) Hmox1 was analyzed using RT-qPCR. Data represent mean ± SEM (boxes) ± SD (whiskers); n=5-7/group. P-values (A-E) obtained using ANOVA followed by LSD post-hoc test. (F) Image of CD163⁺ cells in gut biopsy from a non-T1D control subject (10 year old male) with gastroesophageal reflux disease; bar = 25 µm. (G) Number of CD163⁺ cells/mm² mucosal area in the jejunum of control subjects (filled blue circles; n=14) or patients with type 1 diabetes (red triangles; n=8). Data represent mean ± SD. P-value obtained using Mann-Whitney U-test.

FIG. 6 Gene expression profiling of jejunum identifies Camp upregulation in HC diet-protected rats associated with CD163⁺ M2 macrophages  BBdp rats were maintained under SPF conditions and fed either an HC diet or cereal diet and screened for a panel of innate and adaptive immune factors by PCR array analysis. (A) Results presented as a volcano plot of gene expression in HC-fed rats relative to cereal-fed; circled candidates were either HC-downregulated (green circle) or HC up-regulated (red circles) at least 2-fold with p-values < 0.05; p-values obtained using Student's t-test. (B) Highlighted significant results from the screen indicating Camp and Il1f6 as significant HC up-regulated genes; Lck as a significant HC downregulated gene. (C) Representative images (left panels) displaying CAMP⁺ cells in lamina propria and epithelium of 130 d asymptomatic BBdp jejunum, bar = 20 µm followed by a double immunofluorescence confocal microscopy image displaying CAMP⁺ cell adjacent to CD68⁺ macrophage in lamina propria of a 130 d asymptomatic BBdp rat; bar = 5 µm; CAMP⁺ cells in sterile 130 d germ-free adult BBdp jejunum and BBdp embryonic gut (right panels). (D) Confocal microscopy image displaying multiple CAMP⁺ cells (Cy3/magenta) in the lamina propria of 130 d asymptomatic GF BBdp jejunum co-localizing with both CD14 (Alexa488/green) and CD163 (Cy5/red); nuclei labeled with Hoechst; bars = 5 µm. CAMP⁺CD14⁻CD163⁺ cells (yellow arrows).

FIG. 7  Enteropathy in BBdp rat is not attributable to diabetes risk MHC or Gimap5 mutation  Jejunum sections were from 60-80 d non-diabetes-prone congenic rats (Wistar Furth, BBc, ACI.1⁺, and ACI.1⁺.lyp/lyp) and BBdp rats fed cereal diets. Hematoxylin and eosin stained gut sections displayed crypt hyperplasia, villus atrophy (flattened, shorter), and immune cell infiltration in BBdp rats but not in the non-diabetes-prone rats. All the non-diabetes-prone rats have the diabetes risk MHC and the ACI.1⁺.lyp/lyp rats also have the Gimap5 (lymphopenia) gene but none displays enteropathy, suggesting these genes are not involved in enteropathy and gut inflammation.
Fig. 1

A  Diet and microbes modify diabetes incidence

B  Diet and microbes modify islet homeostasis

C  Dietary modulation of microbial communities
Fig. 2

A  CD3

B  CD8α

C  CD4

Diabetes
**Fig. 3**

**A**

![Image of Foxp3 expression](image)

**B**

![Bar chart showing Foxp3+ cells per mm² mucosa](chart)

**C**

![Bar chart showing Foxp3+ cells / CD³⁺ LPL (%)](chart)

**D**

![Bar chart showing Foxp3+ cells / CD⁴⁺ LPL (%)](chart)
Fig. 4

A. *Il17a* mRNA fold change

B. *Il15* mRNA fold change

C. *Tgfb1* mRNA fold change

D. *Il4* mRNA fold change

E. *Il10* mRNA fold change

F. *Ifng / Il4* mRNA fold change

G. *Ifng / Il10* mRNA fold change

H. *Ifng / Tgfb1* mRNA fold change
Fig. 5
Fig. 6

A  Diet modified genes in jejunum

B  Diet modified Camp, Il1f6 and Lck

C  CAMP distribution in jejunum

D  CAMP in M2 macrophages
Fig. 7

WF

BBc

ACI.1\textsuperscript{u}

ACI.1\textsuperscript{u}.\textit{lyp/lyp}

BBdp

Enlargement of BBdp

\[250\mu m\]

\[250\mu m\]

\[250\mu m\]

\[250\mu m\]

\[250\mu m\]